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*The University of Montana*

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THE COMPARTMENTATION OF CELLULAR PROTEINASES AND INHIBITORS  
IN VEGETATIVE CELLS OF THE CELLULAR SLIME MOLD,  
DICTYOSTELIUM DISCOIDEUM

By

Garrett P. Byrne

B.A., Concordia College, 1983

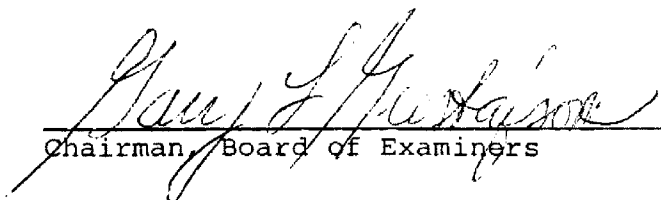
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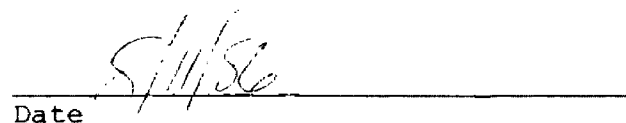
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Byrne, Garrett P., M.S., July 1986

Microbiology

The Compartmentation of Cellular Proteinases and Inhibitors in Vegetative Cells of the Cellular Slime Mold Dictyostelium discoideum. (78 pp.)

Director: Gary L. Gustafson

GLG.

Studies with Proteinase 1 have demonstrated that the enzyme could exist in a masked state in cell homogenates of D. discoideum.\* The masking of Proteinase 1 was attributed to a heat-stable, acid-labile protein inhibitor. During studies to evaluate the compartmentation of Proteinase 1 and Proteinase 2, it was revealed that Proteinase 1 was only present in an active form in the vacuolar fraction. The inhibitor to Proteinase 1, however, was found in the cytosolic fraction. Active Proteinase 2 was not detected in either fraction. Eventually it was determined that Proteinase 2 existed only in the cytosolic fraction and in a masked state. Masked Proteinase 2 was activated by freeze-thaw treatment

It appeared that Proteinase 2 had no apparent function in cells during normal growth and development. Since the masked Proteinase 2 was activated during freeze-thaw treatments, it was suggested that Proteinase 2 could be serving a function related to a stress response. That is, Proteinase 2 could be involved in the degradation of abnormal proteins generated in the cytosol during a stress condition

\*Finn and Gustafson, Manuscript in Preparation

## ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my research advisor Dr. Gary Gustafson for his training and guidance during my graduate studies.

I would also like to thank the members of my committee: Dr. Ralph Judd, Dr. Walter Kooststra, Dr. Walter Hill and Dr. George Card for their critical review of this thesis.

A special thanks is extended to my colleagues in the lab from whom I have learned many things and to my mother Doris Mae Byrne whose constant support and encouragement is one of the most valuable assets I have.

I would like to dedicate this manuscript to Joni, my best friend.

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CHAPTER I  
INTRODUCTION

The cellular slime mold Dictyostelium discoideum is a popular model for the study of molecular processes associated with differentiation. The slime mold is a favorable model because: 1) the differentiation process is rapid and synchronous, and 2) only two cell types are involved during differentiation - stalk cells and spore cells. The studies focused on in this thesis involved the compartmentation of specific cellular proteinases and the evaluation of the inhibition of these proteinases.

I. Life Cycle of the Cellular Slime Mold Dictyostelium discoideum.

The cellular slime mold Dictyostelium discoideum was first reported by Raper in 1935 as a soil amoeba in the woods of North Carolina (1). The amoebae feed on bacteria of decaying leaves and other decomposing matter. The cells divide by binary fission, however upon depletion of bacteria as a food source, the individual amoebae collect into multicellular units and differentiate to form a fruiting body

To initiate differentiation in the laboratory, vegetative cells washed free of bacteria are spread onto a surface of phosphate-buffered agar (2% agar containing 40 mM potassium phosphate, pH 6.6).

About 6-9 hours after differentiation is initiated, cells begin to collect into large streaming patterns to form multicellular aggregates. It has been demonstrated (2) that the aggregation of D. discoideum is induced and controlled by periodic pulses of cAMP (cyclic adenosine monophosphate), which acts as a signalling substance. The formation of streaming patterns is also a regulated process (3) involving two

specific types of contact sites. Contact sites A are involved in end to end adhesion and are stable in EDTA (ethylene diamine tetraacetate). Contact sites B involve side to side adhesion and are EDTA sensitive. These multicellular aggregates become organized into a finger-like stage surrounded by a cellulose sheath. This finger-like structure falls over onto the agar surface becoming a migratory pseudoplasmodium or "slug". This stage develops into a polar body and becomes organized into an area of pre-stalk cells in the anterior region of the slug and an area of pre-spore cells in the posterior region. The cells reach this slug stage 10-14 hours after differentiation is induced. The pseudoplasmodium can migrate for days under favorable conditions, but under laboratory conditions the slug migrates for only a few hours. After the slug stops migrating, the anterior tip (pre-stalk cells) moves vertically in preparation for the culmination stage (16-20 hours after differentiation is induced). Cells at the anterior tip begin to synthesize a ring of cellulose, which extends to form a tubular sheath within the cell mass. Cells within the sheath vacuolize and increase in volume 3-4 fold. When the extending stalk reaches the solid surface, further expansion is restricted to the apical direction. As extension of the stalk continues, cells at the apical tip move into the funnel of the stalk sheath and secrete cellulose forming angular walls. Pre-spore cells at the posterior end begin to travel vertically up the newly formed stalk. Encapsulation of the spore cell begins at the periphery of the rising cell mass (4). As the culmination process proceeds, more and more of the initially posterior cells are encapsulated into heavy walled spores. When all cells have either been engulfed into the stalk



or been encapsulated to form spores, the formation of the fruiting body is complete. The entire process of fruiting body formation is completed 24 hours after differentiation is induced. Stalk cells are no longer viable, but spore cells are capable of enduring long periods of dehydration and temperature changes. Upon reaching a favorable environment, the spore cells germinate, releasing vegetative amoebae.

## II. Proteinase 1 and Proteinase 2 from Dictyostelium discoideum.

### A. Properties of Proteinase 1 and Proteinase 2.

Previous studies have described the isolation and characterization of Proteinase 1 (5,6) and Proteinase 2 (7) from the cellular slime mold Dictyostelium discoideum. Proteinase 1 and Proteinase 2 are the major thiol proteinases, constituting approximately 3% of total cell protein. Proteinase 1 and Proteinase 2 have been shown to share the following properties in common:

#### 1. Proteinase 1 and Proteinase 2 Have Similar Catalytic Properties.

Both Proteinase 1 and Proteinase 2 catalyze the hydrolysis of several model protein substrates such as gelatin and casein. They also hydrolyze the synthetic chromogenic substrate carbobenzoxy-L-lysine-O-nitrophenyl ester (CBZ-Lys-ONp) over a broad pH range (7). The optimal pH for both enzymes was about 5.0. The endopeptidase specificities of Proteinase 1 and Proteinase 2 were compared by the digestion of reduced and carboxymethylated ribonuclease (RCM-RNase). After digestion of RCM-RNase by Proteinase 1 and Proteinase 2, peptide maps were generated by spotting aliquots of the digested material on thin layer plates and developed in the first dimension by electrophoresis (8) and in the

second dimension by partition chromatography. The peptide maps produced were visualized under ultraviolet lamps following treatment of the plates with triethylamine and fluoescamine. Among the 39 peptides generated by digestion of RCM-RNase, 31 were common to both enzymes, demonstrating that the endopeptidase specificities of Proteinase 1 and Proteinase 2 were very similiar.

Both Proteinase 1 and Proteinase 2 were very sensitive to sulfhydryl modifying agents. Storage of the proteinases in the absence of reducing agents, resulted in a gradual loss of esterase activity due to the formation of disulfide crosslinks. However, esterase activity was restored by the addition of 5 mM dithiothreitol (a disulfide reducing agent) to the enzyme preparations. The presence of essential sulfhydryl in the proteinases was also supported by the complete inhibition of enzyme activity by treatments with iodoacetamide or cystamine.

2. Proteinase 1 and Proteinase 2 Contain N-Acetylglucosamine-1-Phosphate Esterified to Peptidyl Serines.

An earlier study demonstrated that Proteinase 1 from the cellular slime mold is conjugated with phosphoryl moieties (9). Subsequent studies (6) demonstrated that all of the phosphate associated with purified Proteinase 1 was released as a sugar-phosphate during mild alkaline treatment. Hydrolysis of Proteinase 1 with 0.4 M NaOH (sodium hydroxide) resulted in the release of low molecular weight phosphoryl moieties from the protein. These moieties were resolved from the enzyme polypeptides by chromatography of the hydrosylates on a column of Sephadex G-25. The total phosphate recovered could be quantitatively hydro-

lyzed to organic phosphate by treatments of the isolated material either: a) with 1N HCl (hydrochloric acid) for 6 minutes in a boiling water bath or b) with alkaline phosphatase. When these hydrosylates were silylated and subjected to GLC (gas liquid chromatography) analysis, only one volatile component was detected. This compound had a retention time identical to the silyl derivative of N-Acetylglucosamine.

The total phosphate present in the material isolated from alkaline hydrosylates of Proteinase 1 migrated as a single phosphoryl compound when subjected to thin layer chromatography. The R<sub>f</sub> value for this compound was identical to that observed for N-Acetyl- $\beta$ -D-Glucosamine-1-Phosphate. Studies have shown (10) that the  $\alpha$  and  $\beta$  anomers of sugar-phosphate could be readily distinguished from each other by their labilities to acid hydrolysis. That is, the  $\beta$ -anomer is much more acid labile than the  $\alpha$ -anomer. Based on these results, it was concluded that the low molecular weight phosphoryl moiety isolated from the alkaline treatments of Proteinase 1 was the  $\alpha$ -anomer of N-Acetylglucosamine-1-Phosphate.

In separate experiments involving acid hydrolysis of Proteinase 1 (6), it was shown that the enzyme phosphate could also be isolated as O-phosphoryl-serine. The release of N-Acetylglucosamine-1-Phosphate from Proteinase 1 during alkaline treatments suggested that residues of the sugar-phosphate might be linked to the peptide structure of Proteinase 1 through phosphodiester bonds to side chain hydroxyls of serine or threonine residues. To examine these possibilities, attempts were made to isolate O-phosphoryl-serine or O-phosphoryl-threonine from acid hydrosylates of Proteinase 1. Purified enzyme was hydrolyzed with

2N HCl under conditions which generally provide for an optimal recovery of these compounds (11) and the hydrosylates were fractionated by thin layer electrophoresis. Two phosphate positive spots were detected on thin layers developed by electrophoresis. One of the spots exhibited a mobility identical to that of O-phosphoryl-serine, the other was determined to be inorganic phosphate. The compound co-migrating with O-phosphoryl-serine was subjected to thin layer chromatography. The results demonstrated that this component had a Rf value identical to that obtained with O-phosphoryl-serine. An aliquot of this component was subjected to quantitative amino acid analysis. This assay detected the presence of 0.41  $\mu\text{mol}$  serine, 0.06  $\mu\text{mol}$  glutamine and less than 0.01  $\mu\text{mol}$  of other amino acids. The calculated ratio of serine:phosphate in the material isolated by electrophoresis was approximately 1:1. It was concluded that the phosphorylated compound isolated was O-phosphoryl-serine. After calculating the percentage loss which occurred during acid hydrolysis of Proteinase 1 and the recovery of O-phosphoryl-serine from thin layer electrophoresis, it was concluded that all of the phosphate in Proteinase 1 was linked to serine residues.

The results of  $^{31}\text{P}$  and  $^{13}\text{C}$  nuclear magnetic resonance (NMR) spectroscopy (12) provided corroborative evidence that essentially all of the N-Acetyl- $\alpha$ -Glucosamine-1-Phosphate residues in Proteinase 1 were esterified to peptidyl serines through phosphodiester bonds. Preliminary  $^{31}\text{P}$  NMR spectroscopy of Proteinase 1 showed only one signal in the general regions where phosphodiesters resonate. Proton-decoupled  $^{13}\text{C}$  NMR spectroscopy of Proteinase 1 showed major signals associated with C1-C6 of the glucosamine residue of O-(N-Acetylglucosamine-1-Phosphoryl) serine residues. Signals were also detected for the methyl carbon

and the carbonyl carbon of the acetyl residue. Resonance signals were also obtained which supported the conclusion that phosphorous was only attached to seryl residues.

A proton-coupled spectrum of Proteinase 1 was obtained and the coupling constant between  $^1\text{H}$  and  $^{13}\text{C}$  at the anomeric carbon was measured. The value determined for this constant confirmed that the anomeric carbon was attached to the aglycan by an  $\alpha$ -linkage.

Quantitative chemical analyses of Proteinase 2 (7) demonstrated that this enzyme also contained N-Acetylglucosamine-1 Phosphate, however, Proteinase 2 contained about 10% as much sugar phosphate as Proteinase 1.

B. Variations in Proteinase Levels as a Function of the Conditions Used for Culturing Dictyostelium discoideum.

Although it has been impossible to grow the wild-type strain (NC-4) of D. discoideum in media without bacteria, a mutant strain has been derived from the NC-4 strain which can grow axenically (13). This mutant strain, termed Ax-3, is capable of growing on bacteria like the NC-4 strain, but it can also be cultured in liquid media which does not contain bacteria. This media contains proteose peptone, yeast extract and glucose.

Lee (14) compared the proteinase levels of the wild-type strain with the proteinase levels of the axenic strain under various growth conditions. It was demonstrated that in Ax-3 cells grown on bacteria, Proteinase 1 predominated over Proteinase 2. The ratio of Proteinase 1 to Proteinase 2 in these cells was about 3:1. This ratio was comparable to the Proteinase 1:Proteinase 2 ratio previously found with NC-4 cells

which was about 2:1. However, Ax-3 cells grown in liquid media contained more Proteinase 2 than Proteinase 1. The ratio of Proteinase 1 to Proteinase 2 in this case was about 1:4.

The issue of how the ratio of proteinases is altered by nutritional environment has not yet been investigated. However, the differences in the ratio of Proteinase:Proteinase 2 in these two groups of cells may be attributed to differences in the nature of polypeptides being utilized as nutrients. Bacterial proteins were mostly highly folded proteins with definite tertiary structure, whereas proteose peptones and peptides from yeast extract presumably contained primarily small peptides. It has been indicated (14) that Proteinase 1 may be more effective than Proteinase 2 in the degradation of protein substrates which have a significant amount of tertiary structure. Therefore it seems likely that bacterial grown cells might have a greater need for Proteinase 1 than axenically grown cells.

C. Secretion of Proteinases During Differentiation of Dictyostelium discoideum.

Studies with the axenic strain of D. discoideum (15) have demonstrated that a variety of lysosomal enzyme are secreted during axenic growth. One group of enzymes including N-Acetylglucosaminidase,  $\alpha$ -mannosidase,  $\beta$ -glucosidase,  $\beta$ -galactosidase and  $\alpha$ -glucosidase-1 were very efficiently secreted with 50-80% of the total cellular activity becoming extracellular within a few hours. However, the secretion of acid phosphatase was distinct from the efficiently secreted glycosidases. It was secreted with linear kinetics for about 6 hours with only 33%

becoming extracellular. This suggested that the secreted acid phosphatase activity made be localized in different vesicles than the above glycosidases. If differentiation was induced, the secretion of these lysosomal enzymes continued through the early stages of differentiation. This secretion appeared to be an active secretory process and not passive leakage of enzyme from the cells. It has been demonstrated that cyanide prevents secretion, implicating an energy requirement normally supplied by electron transport associated with oxidative phosphorylation.

In contrast, wild-type cells do not secrete lysosomal enzymes during growth. However, upon differentiation, Proteinase 1 was secreted only after the stage of late aggregation (16), approximately 6-8 hours after differentiation was induced. The secretion of Proteinase 1 appeared to be selective because other lysosomal enzymes could not be found in the extracellular medium. This suggested a possible separation of Proteinase 1 from other lysosomal enzymes in wild-type cells. The detection of inactive esterase activity in the extracellular differentiation buffer suggested that Proteinase 1 was secreted in a form in which essential sulfhydryls had been oxidized. The current view is that the secretion of Proteinase 1 in wild-type cells was a developmentally regulated process and that the oxidative inactivation of the proteinase may be an integral step in the secretory pathway.

Preliminary studies have demonstrated that Proteinase 2 was also secreted during differentiation of wild-type cells. This appeared to be regulated in a manner similiar to Proteinase 1 secretion.

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### III. Proteinase Inhibitors.

In recent years the field of proteinase inhibitors has received a great deal of attention and it has become increasingly clear that proteinase inhibitors are present in almost every biological system in which proteolysis occurs.

#### A. Inhibitors in Yeast.

Lenney (17) has demonstrated the presence of three unique inhibitors to three known proteases, termed Protease A, B and C in the yeast Saccharomyces cerevisiae. The three proteases are localized as active forms in intracellular vacuoles. The inhibitors to Proteases A, B and C termed Inhibitor A, Inhibitor B and Inhibitor C, respectively, were localized outside the cell vacuole in the cytosol. When yeast cells were disrupted mechanically or by autolysis at pH 7.0, Proteases A, B and C were released from their vacuoles and combined with their respective inhibitors. The inactive form of these proteases were thus enzyme-inhibitor complexes. When the pH was lowered to between 4-6, the proteases digested the inhibitors and then proceeded to non-selectively digest a very high percentage of other yeast proteins. However, because of the localization of proteases and inhibitors in intact cells, protein catabolism probably takes place in the vacuoles.

Since the inhibitors were in a separate compartment from the proteases, it seemed unlikely that the inhibitors served to regulate protease activity. But the high concentration, specificity and affinity of the inhibitors suggested that they play a functional role. Perhaps newly synthesized proteases are transported from cytosolic polysomes to the vacuoles as enzyme inhibitor complexes (17). The other possibility

is that the inhibitors protect extravacuolar proteins in the event of vacuolar leakage.

## B. Inhibitors in Mammalian Systems.

### 1. Rat Liver Thiol Proteinase Inhibitor.

Katunuma has isolated a thiol proteinase inhibitor from rat liver cells (18). This inhibitor was shown to inhibit many thiol proteinases such as Cathepsin H, L, B, C and papain. On subcellular fractionation of rat liver, most of the thiol proteinase inhibitor was recovered in the cytosolic fraction, whereas the inhibitor-sensitive proteinases were recovered from the lysosomal fraction. Amino acid analyses demonstrated that the inhibitor consisted of 98 amino acid residues of which two were cysteines. Upon purification, three forms of the inhibitor were isolated. The three forms of the inhibitor were shown to be due to the oxidation-reduction state of one of the two cysteine residues. It was therefore suggested that the oxidation state may regulate the activity of the thiol proteinase inhibitor in intact cells.

A possible physiological function of the inhibitor has been proposed. Studies suggested that thiol proteinase inhibitors in the cytosolic fraction, function as a safeguard against proteolytic damage due to leakage of lysosomal thiol proteinases.

### 2. Inhibitors in Human Plasma.

Nine different proteinase inhibitors have been identified in human plasma (19). It seemed that most of them functioned similarly in the control of unwanted excessive proteolytic activity in the blood. The inhibitors formed complexes with the proteinases, rendering them

inactive. These complexes were then removed from the circulation by reticuloendothelial cells. One of the major proteinase inhibitors in plasma is  $\alpha_1$ -Antitrypsin ( $\alpha$ AT). Its concentration in plasma is quite high. It was discovered that there was a deficiency in the plasma levels of  $\alpha$ AT in emphysema patients. Experiments carried out to further elucidate the relationship between smoking, proteinase inhibitors and emphysema, found that serum elastase-inhibitory capacity (due mostly to  $\alpha$ AT) was suppressed by cigarette smoke. This suppression was prevented by antioxidants and it was therefore suggested that  $\alpha$ AT was oxidized and rendered inactive and thereby lost its inhibitory capacity

### C. Inhibitors in Plants.

Proteinase inhibitors were recognized in plants about 35 years ago (20) and throughout the years have been considered as possible regulatory or protective proteins. Plant proteinase inhibitors have been demonstrated to function in the regulation of endogenous proteinases of ungerminated seeds such as barley and lettuce. During germination, the inhibitors disappeared before endopeptidase began to increase. These inhibitors appeared to exist only in the resting state of seeds, for they could not be found in any tissues after the onset of germination. It appeared that these inhibitors functioned in the regulation of enzyme activity in dormant seeds.

An entirely different area in which proteinase inhibitors might be of great importance is that of plant protection (21). Tomato leaves contained two proteinase inhibitors. It was found that after pest infestation, that tomato leaves synthesize great amounts of the inhibitors.

It seemed that a proteinase inhibitor inducing factor (PIIF) was produced at the site of plant wounding. This factor spread in the plant and induced the synthesis of inhibitors. Thus, the PIIF appeared to be a plant wound hormone-like substance. Invading pests or mechanical wounding triggers a chain of biochemical events leading to the production of proteinase inhibitors that probably function as part of the plant defense mechanism.

#### D. Inhibitors in Dictyostelium discoideum.

Don Finn in our laboratory has recently detected an inhibitor to Proteinase 1, termed Inhibitor 1 (22). Preliminary studies have demonstrated that the inhibitor is extremely resistant to denaturing agents. After incubation in a boiling water bath or treatment with 8M Guanidine-Hydrochloride, the inhibitor retained its ability to inhibit Proteinase 1. The inhibitor did, however, appear to be sensitive to acidic pH's. An irreversible inactivation of Inhibitor 1 was achieved by incubation at pH 3.0 for 5 minutes at room temperature.

The masking of Proteinase 2 has also been demonstrated. This manuscript will focus on the evaluation of masked Proteinase 2 and the compartmentation of proteinases and inhibitors.

#### IV. Objectives of Thesis Project.

The objectives of this thesis project were the following:

1. To compare the electrophoretic properties of purified Proteinase 1 and Proteinase 2.
2. To evaluate the compartmentation of Proteinase 1 and Proteinase 2 in vegetative cells of D. discoideum.

## CHAPTER TWO

### MATERIALS AND METHODS

#### MATERIALS

A Perkin-Elmer Lambda 3B Spectrophotometer was used for all esterase assays. Sephadex G-200 was purchased from Pharmacia. DE-53 ion exchange resin was purchased from Whatman. Tricine, dithiotreitol, carbobenzoxy-L-Lysine-O-nitrophenyl ester and N-Acetylglucosaminide-ONp were purchased from Sigma. Agar, proteose peptone, yeast extract and acrylamide were purchased from United States Biochemical. Sodium Dodecyl Sulfate was purchased from British Drug House. All other chemicals were reagent grade.

The antisera used in these studies was previously prepared by Don Finn in our laboratory. These antisera had the following specificities:

1) Anti-lb Anti-lb was an antisera generated against the phosphorylated b subunit of Proteinase 1. Subunit b was judged to have the highest content of N-Acetylglucosamine-1-Phosphate based on its staining characteristics in Stains-all.

2) Anti-lb-HF. Anti-lb-HF was an antisera generated against the chemically dephosphorylated b subunit of Proteinase 1. This antisera was judged to be specific for the peptide structure of the b subunit.

3) Anti-P1-N-Acetylglucosamine-1-Phosphate-Affinity Purified. This antisera was generated against the entire N-Acetylglucosamine-1 Phosphate composition of Proteinase 1. The antisera was purified on an affinity column conjugated with Uridine Diphosphate-N-Acetylglucosamine (UDP-GlcNAc)

## METHODS

### I. Culturing Conditions.

The wild-type strain NC-4 of the cellular slime mold Dictyostelium discoideum was used in all of the experiments.

#### A. Preparation and Storage of Spore Plates.

Stock cultures of D. discoideum were maintained as fruiting bodies on nutrient agar plates stored at 4°C. New spore plates were prepared every 4-6 weeks so as to maintain a fresh stock culture of the organism. The nutrient agar used on preparing spore plates contained in 500 ml: 0.5 g yeast extract, 0.5 g peptone, 5 g glucose, 0.48 g K<sub>2</sub>PO<sub>4</sub>, 0.73 g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g MgSO<sub>4</sub> and 10 g agar. This medium was sterilized for 20 minutes at 121°C and poured into petri dishes (approximately 25 ml per dish, dish diameter was 84 mm). The nutrient agar plates were inoculated with: 1) 0.5 ml of a stationary E. coli culture containing 10<sup>8</sup>-10<sup>9</sup> cells/ml and 2) 6-10 spore heads of NC-4 fruiting bodies (obtained from previously prepared spore plates). The mixture of bacteria and spores were spread evenly over the agar surface of each plate. The plates were incubated at 23°C for three days. During these three days of incubation the following events occurred: Day 1, the bacteria multiplied on the plate consuming the available nutrients. Day 2, the bacteria began to be depleted and the confluent lawn of amoebae began to aggregate. Day 3, differentiation of the amoebae was completed with the formation of mature fruiting bodies. The plates containing the mature fruiting bodies were stored at 4°C.

## B. Growth of Cells in Association with Bacteria.

Cells grown in association with Escherichia coli were cultured in covered aluminum pans (16x11x1 inches). Each pan contained 550 ml of sterile nutrient agar medium (the same composition as used for the agar plates). The pans were inoculated with 5 ml of a suspension of E. coli cells and NC-4 spores, prepared by mixing the spores from one spore plate with 20-30 ml of a stationary culture of E. coli. The pans were incubated at 23°C. After approximately 36 hours after incubation, vegetative amoebae were collected and washed free of residual bacteria by repeated centrifugations at 1200 x g in cold 0.5 M Sucrose.

## II. Purification of Proteinases.

All Proteinase 1 and Proteinase 2 preparations employed in this study were previously prepared as described by Gustafson (5). Purified Proteinase 1 was stored in 50% glycerol at -20°C. Purified Proteinase 2 was stored frozen at -20°C.

## III. Assays for Esterase Activity

### A. Assays Employing Sodium Acetate Buffer.

Esterase activity was routinely evaluated based on the enzymes ability to hydrolyze the synthetic chromogenic ester substrate carboxy-benzoyl-L-Lysine-O-Nitrophenyl ester (CBZ-Lys-ONp). A stock solution of the ester substrate was prepared to a final concentration of 5 mM in 50% acetonitrile. Reaction mixtures contained the following: 940 ul of 0.2 M Sodium Acetate·3H<sub>2</sub>O, 5-20 ul of sample, 10 ul of 100 mM dithiothreitol (DTT) and 50 ul of ester substrate. The change in absorbance was followed continuously using a Perkin-Elmer Lambda 3B Spectrophoto-

meter. The CBZ-Lys-ONp exhibited a slow rate of hydrolysis under the conditions used for the assays. The rate of absorbance change was corrected for this non-enzymatic reaction. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the formation of one  $\mu\text{mol}$  of p-nitriphenyl/ minute (extinction coefficient  $5600 \text{ M}^{-1}$ )

#### B. Assays Employing Trichloroacetate Buffer.

Assays for the presence of masked esterase were routinely evaluated by incorporating the chaotropic agent Trichloroacetate (TCA; 1.5 M final concentration) into the assay buffer.

##### 1. Preparation of Assay Buffer Containing 1.6 M Trichloroacetate.

Trichloroacetate (TCA·Na) was prepared immediately prior to use by slowly mixing together equal volumes of 8 M NaOH and 8 M Trichloroacetic acid. The mixing was performed while stirring the solution an ice bath. Sufficient 1 M sodium acetate (NaAc), pH 5.5, was added to the mixture to give a final concentration of 0.2 M NaAc. The solution was diluted with with 0.2 M NaAc (pH 5.5) to give a final TCA·Na concentration of 1.6 M. The pH was adjusted to 5.5 by the dropwise addition of 20-100  $\mu\text{l}$  of 8 M NaOH.

##### 2. Assay Procedure.

The assay for masked esterase was performed by replacing the NaAc buffer described in Part III, A with 1.6 M TCA·Na buffer described above. Reaction mixtures contained 940  $\mu\text{l}$  of TCA·Na buffer (pH 5.5) mixed with 5-20  $\mu\text{l}$  of sample, 10  $\mu\text{l}$  of a 100 mM stock DTT and 50  $\mu\text{l}$  of ester substrate. The fold activation of esterase activity was calculated by dividing the enzyme activity observed in the presence of 1.5 M TCA·Na



by the enzyme activity observed in the presence of 0.2 M NaAc buffer.

C. Assays Employing 0.2 M Glycine-HCl Buffer at pH 3.0.

Preliminary studies have demonstrated that the inhibitor of Proteinase 1 was inactivated under acidic conditions at pH 3.0 (22). 1 ml samples in weakly buffered solutions were mixed with 300 ul of 0.2 M Glycine-HCl (pH 3.0) and incubated for 5 minutes. The samples were brought to a final volume of 2 ml by the addition of 700 ul of 10 mM Tricine (pH 8.0) containing 0.5 mM DTT. Aliquots of this preparation were then assayed for esterase activity in 0.2 M NaAc as described in Part III, A.

D. Assays Employing 0.1% Sodium Dodecyl Sulfate Buffered in Sodium Acetate.

Studies have also shown (22) that Proteinase 1 unmasking was induced in the presence of Sodium Dodecyl Sulfate (SDS). A 1% stock solution of SDS was prepared in 0.2 M NaAc (pH 5.5) buffer. Reaction mixtures contained in 1 ml: 840 ul of 0.2 M NaAc (pH 5.5) mixed with 5-20 ul of sample, 100 ul of 1% SDS stock solution (final concentration of 0.1% SDS), 10 ul of a 100 mM DTT and 50 ul of ester substrate.

IV. Protein Determination

Protein concentrations were estimated spectrophotometrically (23). The optical densities of dilute cell extracts were measured at 260 nm (maximum absorbance for nucleic acids) and 280 nm (maximum absorbance for protein). The protein concentration (mg protein/ml) was the calculated with the following formula:

$$1.45 (OD_{280}) - 0.74 (OD_{260}) = \text{mg protein/ml}$$

## V. Assay for $\beta$ -N-Acetylglucosaminidase

The assay for  $\beta$ -N-Acetylglucosaminidase (a lysosomal glycosidase) was performed essentially as described by Loomis (30). Dilute cytosolic and vacuolar cell extract containing 20-200 ug protein/ml were incubated for 20 minutes at 35°C in 20 mM NaAc (pH 5.5) containing 10 mM p-nitrophenol-N-Acetylglucosaminide. The reaction was stopped after 20 minutes by the addition of an equal volume of 1 M Sodium Carbonate ( $\text{Na}_2\text{CO}_3$ ). The absorbance of the reaction mixtures were measured at 410 nm with a Perkin-Elmer Lambda 3B Spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 umol of p-nitrophenol (extinction coefficient 17,500  $\text{M}^{-1}$ ).

## VI. Electrophoretic Analyses.

### A. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

SDS-PAGE was performed essentially as described by Laemmli (24) in 14 x 16 cm vertical slab gels. The gels consisted of two layers - a separating gel and a stacking gel. The separating gel was prepared to 15% by mixing together the following solutions in a 125 ml Erlenmyer flask: 15 ml of stock acrylamide (30:0.8, acrylamide:bis-acrylamide), 6 ml of 1.875 M Tris buffer (pH 8.8), 0.3 ml of 0.2 M EDTA, 8.4 ml of distilled water, 0.015 ml of TEMED (N,N,N',N'-tetramethylethylenediamine, 99% stock) and 0.3 ml of a 10% ammonium persulfate stock solution. This preparation was mixed thoroughly and approximately 25 ml were poured between the plates of the electrophoresis apparatus. The separating gel was then allowed to polymerize for 20 minutes. During this time, the stacking gel was prepared by mixing the following components in a 125 ml Erlenmyer flask: 2.5 ml of acrylamide (same solution as described above),

1.88 ml of 1 M Tris buffer (pH 6.8), 0.15 ml of 0.2 M EDTA, 10.3 ml of distilled water, 0.0075 ml of TEMED and 0.15 ml of a 10% stock solution of ammonium persulfate. This preparation was mixed well and approximately 8 ml were poured into the electrophoresis apparatus on top of the polymerized separating gel. The desired comb was then inserted into the stacking gel solution and 40-50 minutes were allowed for polymerization.

Running buffer was prepared by combining 3.025 g of Trisma Base, 14.4 g Glycine and 1.0 g of SDS in one liter. The running buffer was distributed equally between the upper and lower reservoirs of the electrophoresis apparatus. Purified samples of Proteinase 1 and Proteinase 2 were mixed with equal volumes of Sample Solubilizing Solution (0.062 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 4% 2-Mercaptoethanol and 0.001% bromophenol blue) and heated at 100°C for 5.5 minutes. The samples were subjected to electrophoresis at 50 ma until the tracking dye (bromophenol blue) had migrated approximately 10 cm (about 4 hours). The gel was removed from the electrophoresis apparatus and stained in Stains-all as described by Green (25).

#### B. Non-Dissociating Polyacrylamide Gel Electrophoresis.

Polyacrylamide gel electrophoresis was performed under non-dissociating conditions essentially as described above. However, 0.1% SDS was deleted from the buffer system. Samples were prepared without 2% SDS and were not heat treated. Samples were subjected to electrophoresis at 50 ma for 4 hours. The gel was removed from the electrophoresis apparatus and stained as described above.

## VII. Caseogram

The procedure described by Foltman (26) was used for detecting proteinases after gel electrophoresis. Proteinases were first subjected to electrophoresis under non-dissociating conditions taking special precautions to retain proteolytic activity. Following electrophoresis, the gel was layered onto a glass plate coated with 1% agarose containing 1% skim milk powder. After incubation at room temperature for two hours, the proteinase containing zones produced distinct precipitates in the skim milk gel.

### A. Non-Dissociating Phosphate-Buffered PAGE.

An alternate method for non-dissociating gel electrophoresis was performed. A phosphate-buffered, 12.5% acrylamide gel was prepared by combining the following: 17.4 ml of 100 mM phosphate buffer, 14.6 ml of acrylamide (same composition used in the Laemmli system in Part VI,A), 0.35 ml of a 10% stock solution of ammonium persulfate, 2.7 ml of distilled water and 0.0175 ml TEMED. Approximately 30 ml of this solution was poured into a 14 x 16 cm electrophoresis apparatus and allowed to polymerize for 40 minutes (no stacking gel was used). The running buffer was 50 mM sodium phosphate. To maintain proteolytic activity during electrophoresis, 0.5 mM DTT and 2.5 mM glutathione were added to the running buffer of the upper reservoir of the electrophoresis apparatus. To eliminate contact between the proteinase preparations and ammonium persulfate (a strong oxidant) the gel was developed with a current of 75 ma for 30 minutes before samples were applied. The samples were subjected to electrophoresis for approximately 5 hours. The gel was carefully removed from the electrophoresis apparatus and equilibrated in 0.2 M NaAc (pH 5.5)

for 3 buffer changes at 30 minutes each.

#### B. Preparation of Casein-Agarose Plates.

Casein plates were prepared in the following stepwise fashion: Step 1, 16 x 18 cm glass plates were pre-coated with a solution of 0.1% agarose in 0.2 M NaAc (pH 5.5) containing 0.02% sodium azide and allowed to dry. The plates were then placed in a 60°C incubator for 20 minutes. Step 2, A 1% agarose solution containing 1% skim milk powder was prepared in 0.2 M NaAc (pH 5.5) containing 0.02% sodium azide. This solution was heated in a water bath to 55-60°C. Step 3, Approximately 25 ml of this solution was layered onto each pre-coated, pre-heated glass plate to an even thickness of 1.5 mm. The casein plates were kept at 4°C for at least 15 minutes.

#### C. Development of Caseograms.

The electrophoresis gel was removed from the equilibration buffer. Contact was made along one edge of the casein-agarose plate and the gel was carefully rolled onto the surface. The sandwiched gels were then covered with clear cellophane, taking care to avoid air bubbles, and incubated at room temperature under conditions of high humidity. Incubation for two hours was generally sufficient for development. To unmask proteolytic activity, the gel was carefully removed from the casein-agarose plate and incubated for 30 minutes in 0.2 M NaAc (pH 5.5) containing 0.1% SDS. After incubation, the gel was layered onto a new casein-agarose plate and again incubated at room temperature for 2 hours.

## VIII. Immunological Analyses.

### A. Electroblotting

Blotting buffer was prepared as a 10x stock solution (0.2M sodium phosphate, pH 7.4) and was diluted immediately prior to use to a final concentration of 0.02 M phosphate (400 ml of stock blotting buffer was mixed with 3600 ml of distilled water). 2500 ml were poured into a water cooled electroblotting apparatus (dimensions 10x20x28 cm) and cooled for two hours. The remaining 1500 ml were used to equilibrate the nitrocellulose membrane required for blotting.

Proteins for immunological studies were first resolved on a 15% SDS-PAGE gel. The gel was then removed from the electrophoresis apparatus and rinsed with distilled water. The gel was layered onto a sheet of nitrocellulose membrane (10 x 15 cm) previously equilibrated in the 0.02 M sodium phosphate buffer. The gel and the nitrocellulose membrane were sandwiched between two pieces of filter paper. This sandwich was placed between two Scotch-Brite pads and inserted into a plastic holder. This entire assembly was then placed into the blotting chamber containing the blotting buffer. Proteins were electrophoretically transferred to the nitrocellulose membrane (toward the positive electrode) at 24 volts for 15 hours.

### B. Reactions of Electroblots with Specific Antisera.

The nitrocellulose membrane was removed from the electroblotting chamber and equilibrated for 1 hour in TTBS (20 mM Tris-buffered saline, pH 7.4, containing 0.05% Tween-20) and then probed with either a 1:300 dilution of Anti-Ib, 1:100 dilution of Anti-Ib-HF or a 1:600 dilution of Anti-P1-N-Acetylglucosamine-1-Phosphate-Affinity Purified Antibody

for a 2 hour incubation. All antisera dilutions were in TTBS. After incubation, the membrane was rinsed with three changes of TTBS for 10 minutes each, followed by incubation for 1 hour with a 1:300 dilution of the antibody conjugate Goat-Anti-Rabbit-Horse Radish Peroxidase (GAR-HRP). After incubation, the nitrocellulose membrane was again rinsed with three changes of buffer, however, the last 10 minute rinse was in TBS (Tris buffered saline: TTBS without Tween-20). Developer was prepared immediately prior to use by adding 0.66 mg of 4-Chloro-1-Naphthol to 20 ml of cold methanol. This was mixed with 100 ml of TBS containing 60  $\mu$ l of  $H_2O_2$  (8.8 M). This solution acted as a substrate for the conjugated Horse Radish Peroxidase. The nitrocellulose membrane was incubated in this solution until optimal development occurred (usually 15-20 minutes). The reaction was stopped by repeated rinsing in distilled water.

#### IX. The Isolation of Cytosolic and Vacuolar Fractions.

The isolation of cytosolic and vacuolar fractions was performed as follows: A. Vegetative cells of D. discoideum were harvested after depletion of bacteria but prior to the onset of cellular aggregation. B. Cells were collected and washed free of residual bacteria by repeated centrifugations in cold 0.5 M Sucrose. C. Washed cells were suspended to a cell density of  $1.0 \times 10^9$  cells/ml and subjected to 40 strokes of homogenization in a Dounce homogenizer (Wheaton Scientific Co., Millville, NJ.) D. The cell homogenate was mixed with an equal volume of cold 0.5 M Sucrose and centrifuged at  $1200 \times g$  for 5 minutes to remove whole cells. E. The pellet of undisrupted cells was discarded and the supernatant fluid was centrifuged at  $15,000 \times g$  for 30 minutes.

F. The supernatant fluid (cytosolic fraction) was decanted and the pellet (vacuolar fraction) was washed with 0.5 M sucrose and suspended in a small volume of distilled water. This osmotic shock provided for the disruption of lysosomes and other vacuoles. The cytosolic fraction was exhaustively dialyzed against 10 mM Tricine (pH 8.0), 0.5 mM DTT and centrifuged at 240,000 x g for 60 minutes. The resulting supernatant was then concentrated by pressure dialysis to a volume of 1-2 ml in an Amicon ultrafiltration cell (Amicon Corp., Lexington, Mass.) equipped with a YM-10 filter.



## CHAPTER III

### RESULTS

#### I. Proteinase 1 and Proteinase 2 from Whole Cell Extracts of Dictyostelium discoideum.

Previous studies (5,7) have employed gel exclusion and ion exchange chromatography to resolve Proteinase 1 and Proteinase 2 from whole cell extracts of D. discoideum. When a whole cell extract was applied to a column of Sephadex G-200, esterase activity eluted from the column in two peaks, Figure 1A. The large peak at a relative elution volume ( $V_e/V_o$ ) of 1.6 was Proteinase 1 and the smaller peak at a relative elution volume of 2.2 was Proteinase 2. Proteinase 2 had a lower native molecular weight than Proteinase 1.

Panel B shows the resolution of Proteinase 1 and Proteinase 2 on a column of Whatman DE-53 cellulose. The column was developed with a linear salt gradient from 0.1M to 0.5M. Esterase activity eluted from the column in two peaks. Proteinase 1 eluted from the column at a salt concentration of 0.31M and Proteinase 2 eluted from the column at 0.24M.

#### II. Comparison of Proteinase 1 and Proteinase 2 with PolyAcrylamide Gel Electrophoresis.

Gustafson and Thon (5) showed that Proteinase 1 was a polymeric protein with three kinds of subunits. Finn demonstrated (16) that the three subunits could be resolved by Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE). As shown in Figure 2, the three subunits designated as a, b and c had apparent molecular weights of 55, 39 and 15 kilodaltons, respectively. When purified Proteinase 2 was dissociated and subjected to SDS-PAGE, one major polypeptide was resolved with a molecular weight of approximately 20 kilodaltons. A minor band was also detected which had a molecular weight similar to the b subunit of

Figure 1      Panel A.    Sephadex G-200 Chromatography of a Crude Cell Extract of D. discoideum.

Two trays of vegetative amoebae of D. discoideum were harvested in 0.5 M sucrose. Washed cells were suspended to a cell density of  $5.0 \times 10^8$  cells/ml in 10 mM Tricine (pH 8.0), 0.5 mM DTT and stored frozen at  $-20^{\circ}\text{C}$ . A 1 ml aliquot of the cell suspension was applied to a column (1.7 x 40 cm) of Sephadex G-200, previously equilibrated in 10 mM Tricine (pH 8.0), 0.5 mM DTT. The column was eluted with the above buffer and 2 ml fractions were collected. Fractions were assayed for esterase activity.

Panel B.    DEAE Cellulose Chromatography of a Crude Cell Extract of D. discoideum.

Two trays of vegetative amoebae of D. discoideum were harvested in 0.5 M sucrose. Washed cells were suspended to a cell density of  $5.0 \times 10^8$  cells/ml in 10 mM Tricine (pH 8.0), 0.1 M NaCl, 0.5 mM DTT and stored at  $-20^{\circ}\text{C}$ . A 1 ml aliquot was suspended in 10 ml of the above buffer and applied to a column (1.5 x 6.0 cm) of Whatman DE-53 cellulose Previously equilibrated in 10 mM Tricine (pH 8.0), 0.1 M NaCl, 0.5 mM DTT. The column was eluted with the above buffer, followed by elution with a 60 ml linear NaCl gradient from 0.1 M to 0.5 M (in the same buffer). Two ml fractions were collected. Fractions were assayed for esterase activity.

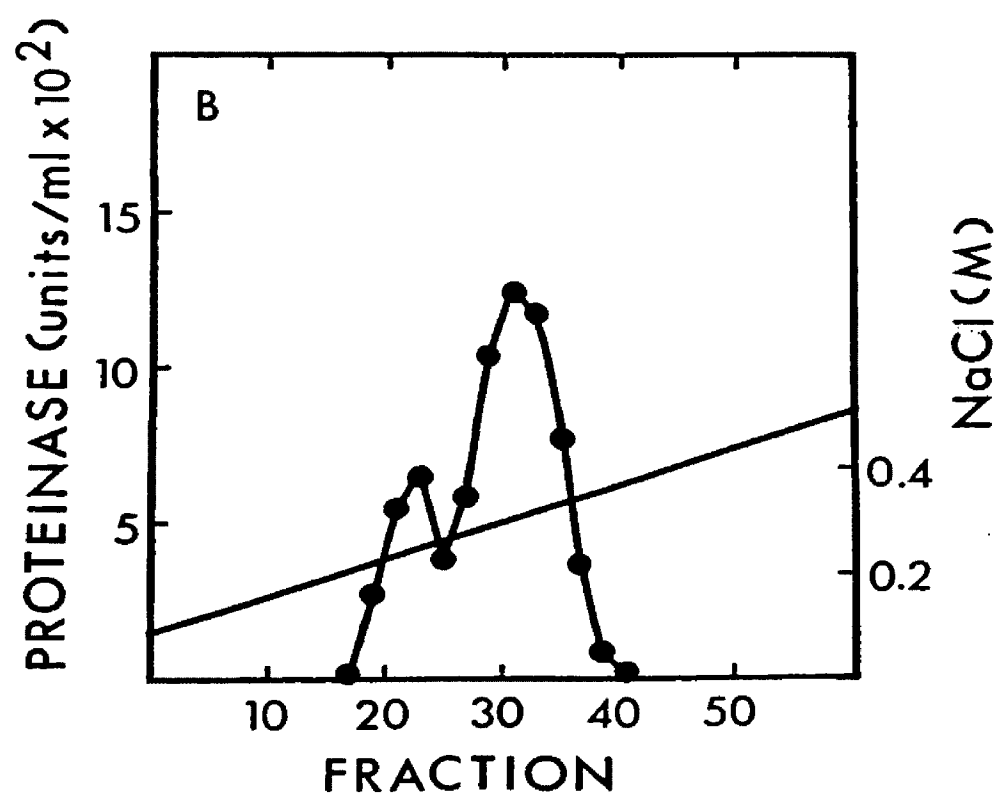
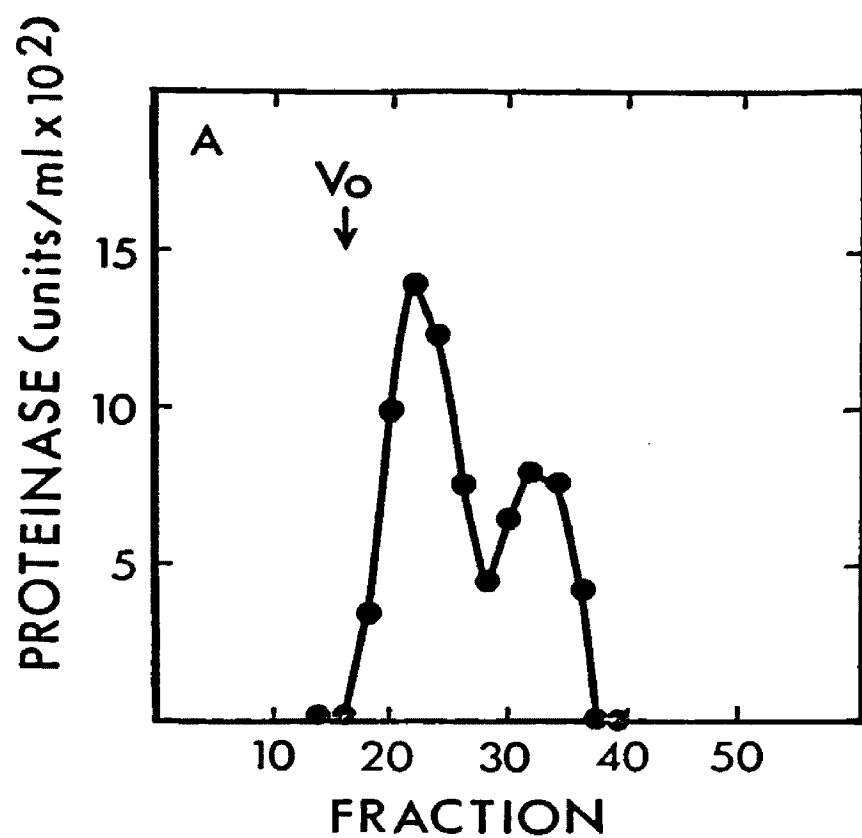
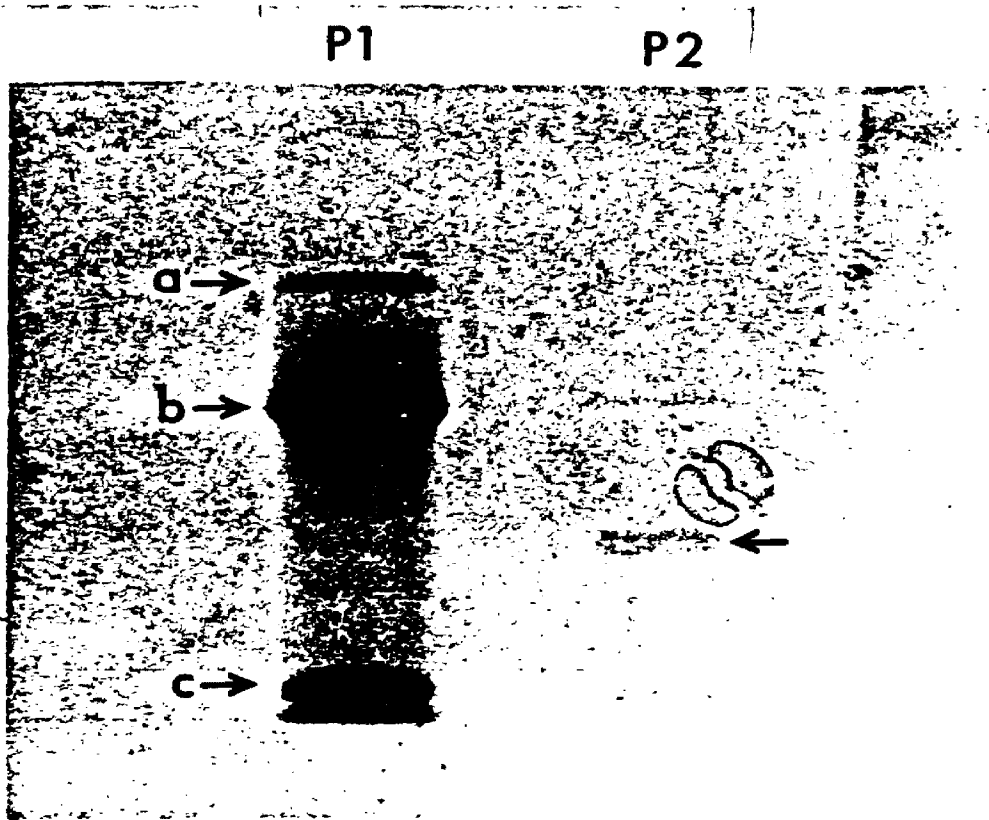


Figure 2      SDS-PAGE of Purified Proteinase 1 and Proteinase 2

Purified samples of Proteinase 1 and Proteinase 2 were subjected to electrophoresis in a 15% SDS-PAGE gel as described in the Methods section VI, A. The gel was removed from the electrophoresis apparatus and stained in Stains-all.



## Proteinase 1.

Proteinase 1 and Proteinase 2 were also prepared under non-dissociating conditions and subjected to non-dissociating PAGE. The results in Figure 3 showed that both Proteinase 1 and Proteinase 2 resolved as single major bands. The native form of Proteinase 2 had a greater mobility than Proteinase 1 under these conditions

### III. Immunological Properties of Proteinase 1 and Proteinase 2.

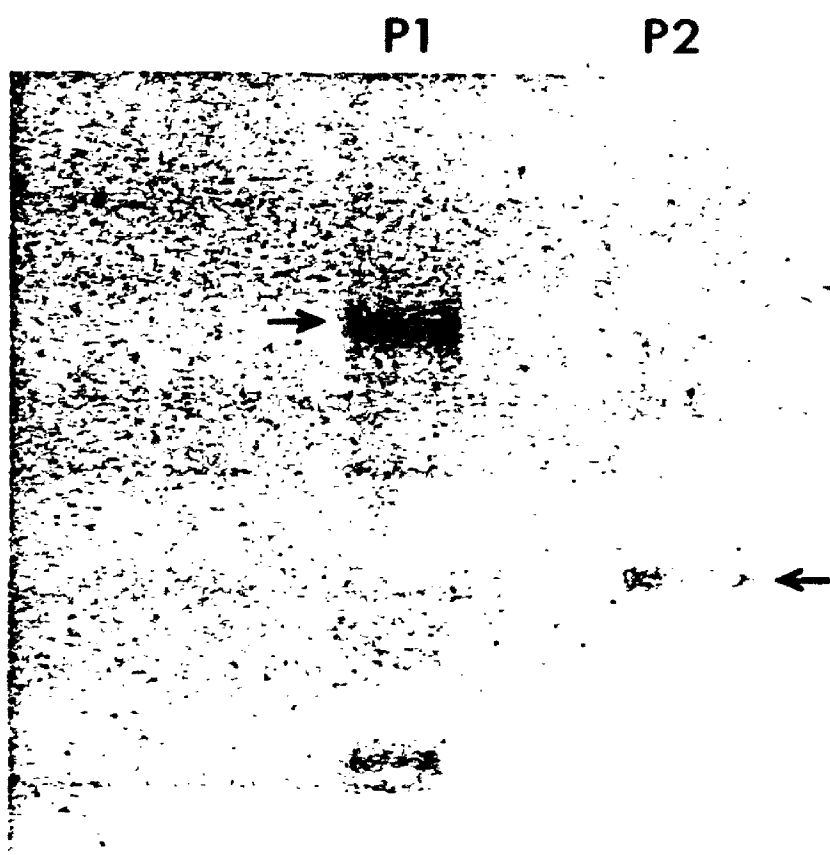
The specificities of antisera generated against the phosphorylated subunit of Proteinase 1 (Anti-lb) and the chemically dephosphorylated subunit (Anti-lb-HF) of Proteinase 1 (see Materials) were compared in their reactions with purified Proteinase 1 and Proteinase 2. For these analyses, Proteinase 1 and Proteinase 2 were first resolved by SDS-PAGE then electroblotted to a nitrocellulose membrane. Antigen reacting with these probes were labeled by the enzyme linked antibody probe described in the Methods section. Finn has previously shown (16) that Anti-lb reacted with determinants in all three subunits of Proteinase 1 where as Anti-lb-HF recognized determinants unique to the b subunit of Proteinase 1. Figure 4 shows that the Proteinase 2 polypeptide cross-reacted with both of these antisera. These results supported the view that Proteinase 2 contained polypeptide determinants that were similiar to determinants in the b subunit of Proteinase 1.

### IV. Compartmentation of Proteinase 1 and Proteinase 2.

In the original studies of Proteinase 1 and Proteinase 2 by Gustafson and Thon (5), the proteinases were extracted from cells that had been disrupted by freezing and thawing. Since this treatment disrupted cell

**Figure 3**      **Non-dissociating PAGE of Purified Proteinase 1 and Proteinase 2.**

Purified samples of Proteinase 1 and Proteinase 2 were subjected to electrophoresis in a 15% non-dissociating gel as described in the Methods section VI, B. The gel was removed from the electrophoresis apparatus and stained in Stains-all.





**Figure 4**      Comparison of Purified Proteinase 1 and Proteinase 2  
by Their Reactions with Specific Antisera.

Purified samples of Proteinase 1 and Proteinase 2 were subjected to electrophoresis in a 15% SDS-PAGE gel followed by electrophoretic transfer to a nitrocellulose membrane (see Methods VIII, A and B). The nitrocellulose membrane was then reacted with a 1:300 dilution of Anti-lb antisera (Panel A) or a 1:100 dilution of Anti-lb-HF (Panel B).

	<b>A</b>		<b>B</b>
<b>P1</b>	<b>P2</b>	<b>P1</b>	<b>P2</b>

**a**

**b**

**b**

**c**

vacuoles, the lysosomal proteinases were isolated in mixture with cytosolic proteins. To evaluate the compartmentation of Proteinase 1 and Proteinase 2, cytosolic and vacuolar preparations were obtained from a cell extract which had not been subjected to freezing and thawing. The cytosolic and vacuolar fractions were then individually analyzed for esterase activity.

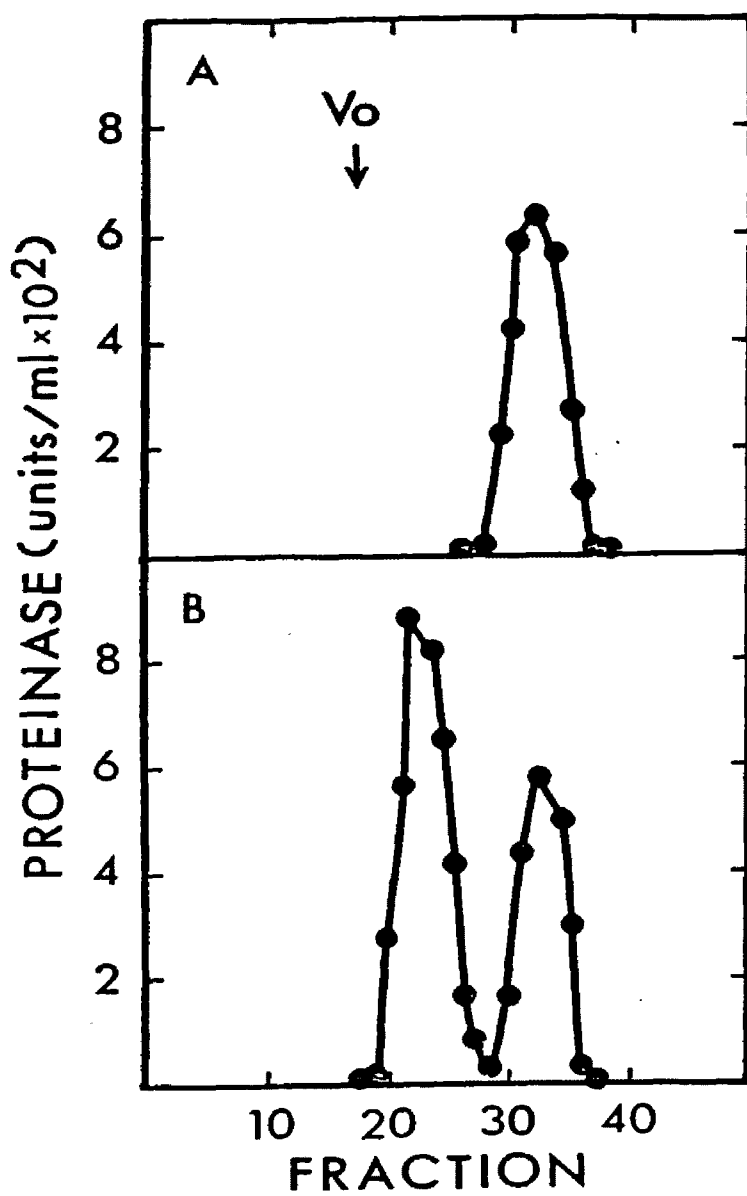
Early in these studies, it was observed that the esterase activity present in a cytosolic fraction increased 4-5 fold when the fraction was subjected to freezing and thawing at  $-20^{\circ}\text{C}$ . Cytosolic esterase was examined by applying a frozen-thawed preparation to a column of Sephadex G-200. A single peak of active esterase eluted from the column, Figure 5. This peak of esterase activity corresponded to the elution characteristics of Proteinase 2 which had been previously examined (see Figure 1A).

Studies by Finn (22) have shown that Proteinase 1 can exist in a masked state. The masking of Proteinase 1 has been attributed to a heat-stable, acid-labile protein inhibitor. To evaluate the possible presence of masked Proteinase 1 in the cytosol, the column fractions were incubated at pH 3.0 for 5 minutes (these conditions were found favorable for the unmasking of Proteinase 1). Panel B of Figure 5 shows that this treatment unmasked enzyme activity corresponding to Proteinase 1.

In order to further investigate if the freeze-thaw activated proteinase corresponded to Proteinase 2, ion exchange chromatography was performed. The remaining volumes of the column fractions containing this peak of esterase activity (from the experiment in Figure 5) were pooled and applied to a column of Whatman DE-53 cellulose. The column was eluted with a

Figure 5      Sephadex G-200 Chromatography of a Frozen-Thawed Cytosolic Extract Followed by Acid Treatment of the Column Fractions.

One tray of vegetative amoebae of *D. discoideum* was harvested in 0.5 M sucrose. The cells, washed free of bacteria, were suspended to a cell density of  $5.0 \times 10^8$  cells/ml in 0.5 M sucrose. The cytosolic extract was obtained by homogenization and centrifugation (see Methods section IX). This concentration was concentrated to a volume of 1-2 ml and frozen at  $-20^{\circ}\text{C}$ . The thawed material was applied to a column (1.7 x 40 cm) of Sephadex G-200, previously equilibrated in 10 mM Tricine (pH 8.0), 0.5 mM DTT and eluted with the same buffer. Two ml fractions were collected and 0.5 ml of each fraction was assayed for esterase activity (Panel A). One ml of the remaining volumes of the column fractions were incubated at pH 3.0 (Methods III, C) for 5 minutes and assayed for esterase activity (Panel B).



linear salt gradient. A single peak of esterase activity eluted from the column at a salt concentration of approximately 0.24M, Figure 6. This peak corresponded to the elution characteristics of Proteinase 2 that had been examined in whole cell extracts (Figure 1B).

Figure 7 shows an alternative approach that was used to examine the selective unmasking of proteinases. It was demonstrated in Figure 5 that a cytosolic esterase corresponding to Proteinase 1 could be selectively unmasked after the resolution of the enzyme on a Sephadex G-200 column. The results presented in Figure 7 showed that the esterase corresponding to Proteinase 2 could also be selectively unmasked in chromatography fractions. In this experiment, a cytosolic fraction was subjected to acid treatment at pH 3.0 and applied to a column of Sephadex G-200. Esterase activity eluted from the column as a single peak which corresponded to Proteinase 1. The chromatography fractions were then frozen at  $-20^{\circ}\text{C}$ , thawed and assayed for esterase activity. Panel B of Figure 7 demonstrated that esterase activity corresponding to Proteinase 2 was unmasked upon conditions of freezing and thawing.

To determine if both proteinases could be unmasked prior to chromatography, a cytosolic preparation was incubated at pH 3.0 for 5 minutes then frozen at  $-20^{\circ}\text{C}$ . This preparation was thawed and divided into two equal parts, A and B. Part A was applied to a column of Sephadex G-200. Esterase activity eluted from the column in two peaks, Figure 8A. The peaks corresponded to the elution characteristics of Proteinase 1 and Proteinase 2 (see Figure 1A). Part B was applied to a column of Whatman DE-53 cellulose. Esterase activity also eluted from the column in two peaks. These peaks again corresponded to the elution characteristics of Proteinase 1 and Proteinase 2 (see Figure 1B).

**Figure 6**      **DEAE Cellulose Chromatography of the Low Molecular Weight Proteinase Present in a Frozen-Thawed Cytosolic Extract.**

The column fractions containing the low molecular weight proteinase (from Figure 5) were collected, pooled and concentrated to a volume of 1-2 ml. This solution was equilibrated in 10 ml of 10 mM Tricine (pH 8.0), 0.1 M NaCl and 0.5 mM DTT. The preparation was then applied to a column (1.5 x 6.0 cm) of Whatman DE-53 cellulose previously equilibrated in the above buffer. The column was eluted with 20 ml of buffer followed by elution with 60 ml of a linear NaCl gradient from 0.1 M to 0.5 M (in the same buffer). One ml fractions were collected. Fractions were assayed for esterase activity.

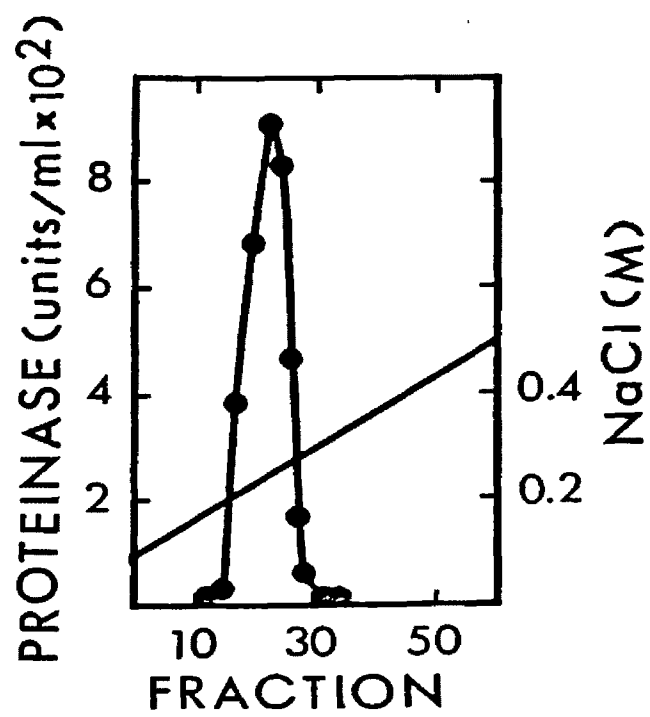
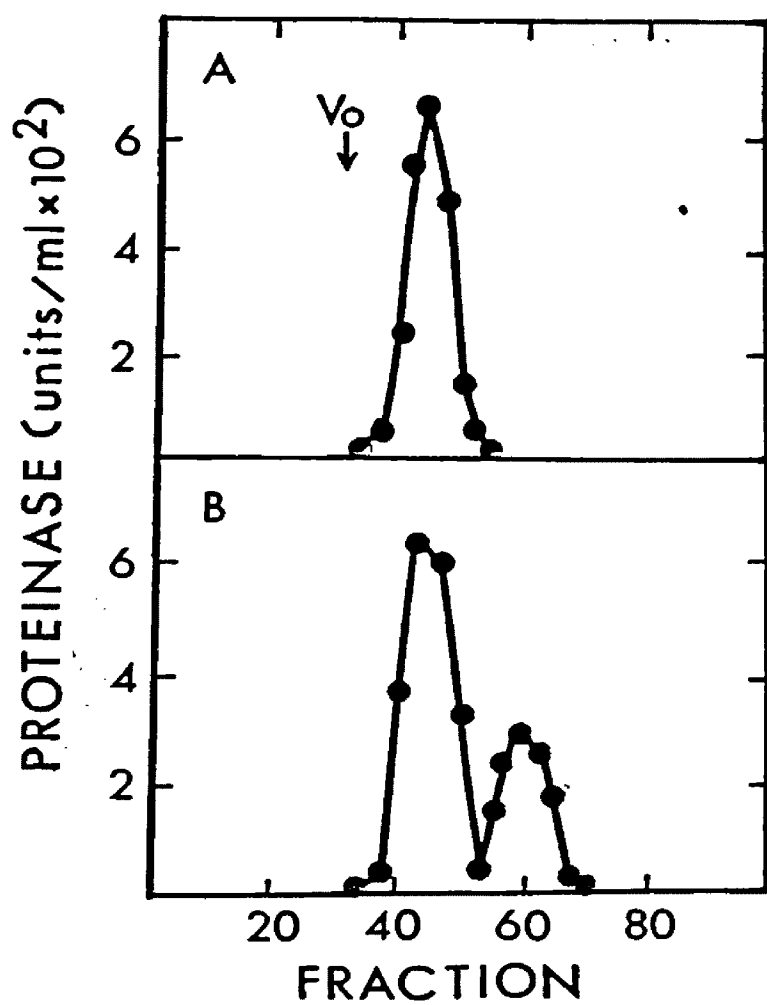




Figure 7      Sephadex G-200 Chromatography of An Acid Treated Cytosolic Extract Followed by Freeze-Thaw Treatment of the Column Fractions.

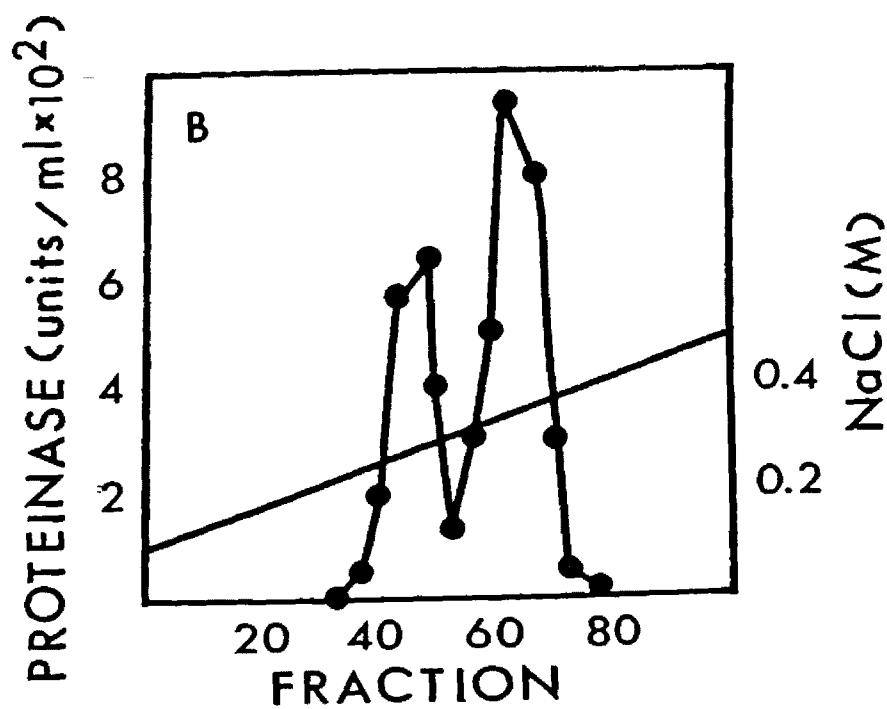
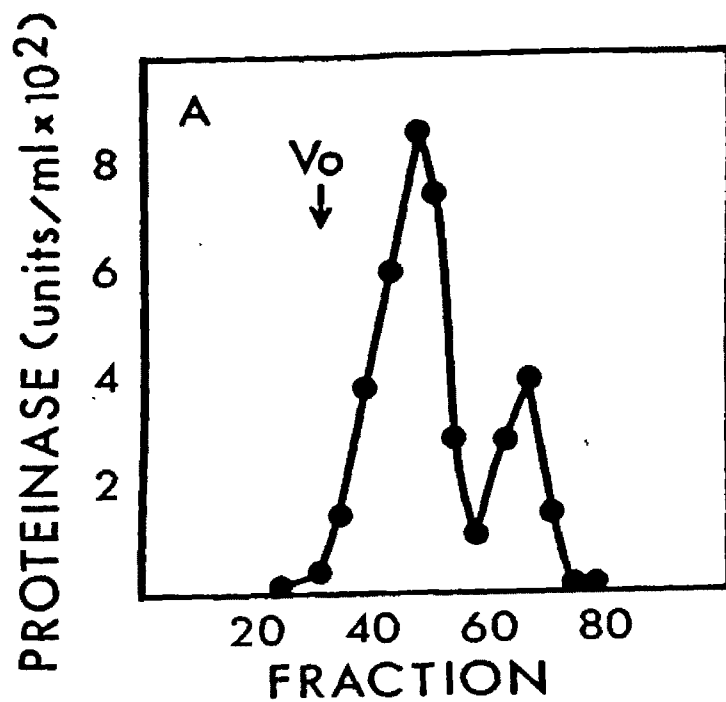
One tray of vegetative amoebae of D. discoideum were harvested in 0.5 M sucrose. The cells, washed free of bacteria, were suspended to a cell density of  $5.0 \times 10^8$  cells/ml in 0.5 M sucrose. The cytosolic extract was obtained by homogenization and centrifugation and concentrated to a volume of 1-2 ml. One ml of this preparation was subjected to acid treatment at pH 3.0 for 5 minutes. This material was then applied to a column (1.7 x 40 cm) of Sephadex G-200, previously equilibrated in 10 mM Tricine (pH 8.0), 0.5 mM DTT and eluted with the same buffer. One ml fractions were collected and 0.5 ml of each fraction was assayed for esterase activity (Panel A). The remaining 0.5 ml of each fraction was frozen at  $-20^{\circ}\text{C}$  overnight, thawed and assayed for esterase activity (Panel B0).



**Figure 8**      **Sephadex G-200 Chromatography and DEAE Cellulose Chromatography of a Frozen-Thawed and Acid Treated Cytosolic Extract.**

One tray of vegetative amoebae of D. discoideum was harvested in 0.5 M sucrose. The cells, washed free of bacteria, were suspended to a cell density of  $5.0 \times 10^8$  cells/ml in 0.5 M sucrose. The cytosolic extract was obtained by homogenization and centrifugation and concentrated to a volume of 1-2 ml. This preparation was frozen at  $-20^{\circ}\text{C}$  overnight, thawed and one ml of this solution was subjected to acid treatment at pH 3.0 for 5 minutes. This material was divided into two equal parts, A and B. Part A was applied to a column (1.7 x 40 cm) of Sephadex G-200 previously equilibrated in 10 mM Tricine (pH 8.0), 0.5 mM DTT and eluted with the same buffer. One ml fractions were collected. Fractions were assayed for esterase activity (Panel A).

Part B was equilibrated in 10 ml of 10 mM Tricine (pH 8.0), 0.1 M NaCl, 0.5 mM DTT and applied to a column (1.5 x 6.0 cm) of Whatman DE-53 cellulose previously equilibrated in the above buffer. The column was eluted with buffer, followed by elution with a 60 ml linear NaCl gradient from 0.1 M to 0.5 M. One ml fractions were collected. Fractions were assayed for esterase activity (Panel B).

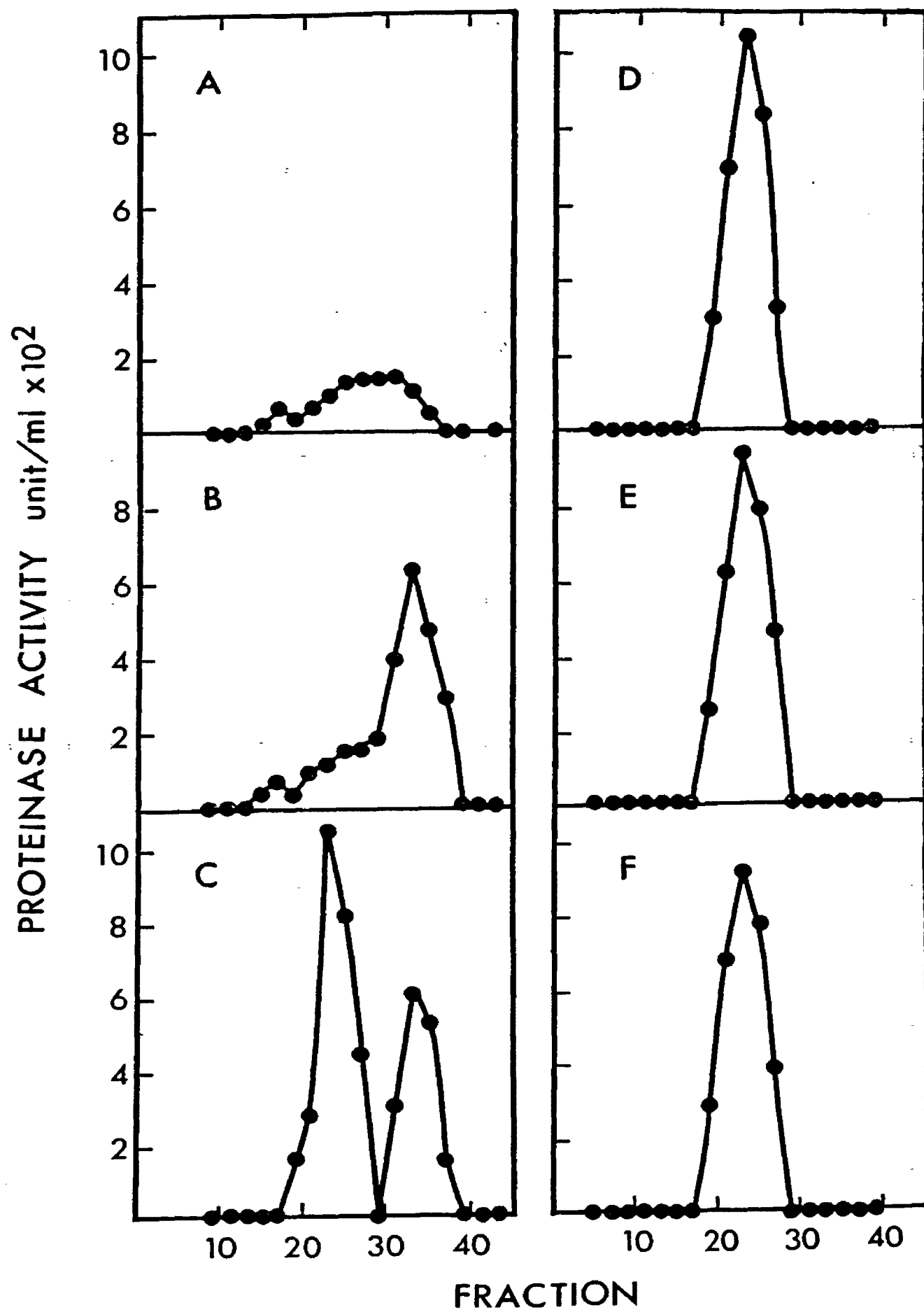


In the previous experiments (Figures 5 and 7), one of the proteinases in the cytosolic fraction was unmasked prior to chromatography. In contrast, Figure 9 shows a study where both proteinases were first resolved in their masked forms and each was then selectively unmasked by freeze-thaw and acid treatments. Panel A reveals that no significant esterase activity was present in an untreated cytosolic extract. However, when the column fractions were frozen at  $-20^{\circ}\text{C}$ , thawed and assayed, esterase activity corresponding to Proteinase 2 was unmasked (Panel B). Incubation of the remaining volumes of the column fractions from Panel B at pH 3.0 for 5 minutes, reversed the inhibition of Proteinase 1 thus activating Proteinase 1 esterase activity (Panel C). It was also possible to reverse the order of treatment and unmask the esterase activity corresponding to Proteinase 1 first by acid incubation followed by freeze-thaw activation of the esterase corresponding to Proteinase 2 (data not shown).

In parallel with the analyses of the cytosolic proteinases, we also examined how freeze-thaw and acid treatments influenced the activity of the proteinases associated with the vacuolar fraction. Panel D revealed a peak of esterase activity corresponding to Proteinase 1. Freezing and thawing of the column fractions did not provide for unmasking of any additional proteinase activity (Panel E). Subsequent acid treatment of the fractions from Panel E also did not unmask any additional esterase activity (Panel F). These results suggested that the lysosomes contained only active Proteinase 1 but no active or masked Proteinase 2.

Figure 9      Sephadex G-200 Chromatography of Cytosolic and Vacuolar Fractions Followed by Freeze-Thaw and Acid Treatment of the Column Fractions.

Two trays of vegetative amoebae of *D. discoideum* were harvested in 0.5 M sucrose. The cells, washed free of bacteria, were suspended to a cell density of  $5.0 \times 10^8$  cells/ml in 0.5 M sucrose. The cytosolic and vacuolar fractions were obtained by homogenization and centrifugation and were individually applied to a column (1.7 x 40 cm) of Sephadex G-200, previously equilibrated in 10 mM Tricine (pH 8.0), 0.5 mM DTT and eluted with the same buffer. Two ml fractions were collected. The left side of the figure are the results of the chromatography of the cytosolic fraction. 0.5 ml of the column fractions were assayed for esterase activity (Panel A). Small aliquots (0.5 ml) of each fraction was then stored at  $-20^{\circ}\text{C}$  overnight, thawed and assayed for esterase activity (Panel B). The remaining 1 ml of each fraction was subjected to acid treatment at pH 3.0 for 5 minutes and then assayed for esterase activity (Panel C). The column fractions from the chromatography of the vacuolar fraction were treated in a similar manner. 0.5 ml of each fraction was assayed for esterase activity (Panel D). The column fractions were then subjected to freeze-thaw (Panel E) and acid treatment (Panel F).



## V. Comparison of the Compartmentation of Proteinase 1 with the Compartmentation of $\beta$ -N-Acetylglucosaminidase.

The purpose of the studies in this section was to examine whether the presence of Proteinase 1 in the cytoplasmic fractions was a result of the partial disruption of some vacuoles during the process of cell homogenization. To examine this possibility, the proportion of Proteinase 1 in the cytosol was compared to the proportion of  $\beta$ -N-Acetylglucosaminidase in the cytosol. Studies by Ashworth (27) and Dimond (15) have shown that  $\beta$ -N-Acetylglucosaminidase is a glycosidase present in the lysosomal compartment of D. discoideum. If no disruption of vacuoles occurred during homogenization, then  $\beta$ -N-Acetylglucosaminidase would not be detected in the cytosol. The data presented in Table 1 revealed that 23% of the total  $\beta$ -N-Acetylglucosaminidase in the cells was present in the cytosol after homogenization, compared to 16% of the total Proteinase 1 activity. These results suggested that the vacuoles were partially disrupted during homogenization which could account for the presence of Proteinase 1 in the cytosol.

## VI. The Isolation of the Freeze-Thaw Activated Proteinase.

DE-53 cellulose and gel exclusion chromatography were combined to isolate the freeze-thaw activated proteinase from the cytosol of vegetative cells. A cytosolic preparation was obtained from 10 trays of vegetative amoebae and applied to a column of Whatman DE-53 cellulose, followed by elution with a linear salt gradient from 0.1M to 0.5M. Small volumes of the resulting chromatography fractions were then subjected to freeze-thaw treatment at  $-20^{\circ}\text{C}$ . Figure 10 shows that a single peak of esterase activity was unmasked with this treatment.



Table 1: Distribution of Proteinase 1 and  $\beta$ -N-Acetylglucosaminidase in Cell Homogenates in *D. discoideum*.

<u>Enzyme</u>	<u>Total Units in Vacuolar Fraction</u>	<u>Total Units in Cytosolic Fraction</u>	<u>Percent Enzyme Activity in Cytosolic Fraction</u>	<u>Percent Enzyme Activity in Vacuolar Fraction</u>
Proteinase 1	78.0	12.4*	16%	84%
$\beta$ -N-Acetyl- glucosamin- idase	7.8	1.8	23%	74%

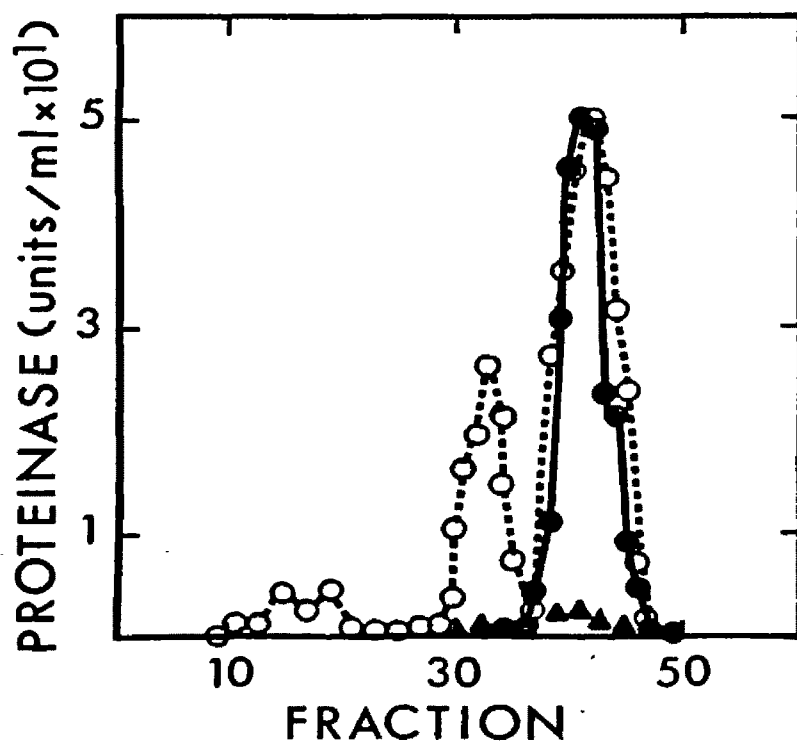
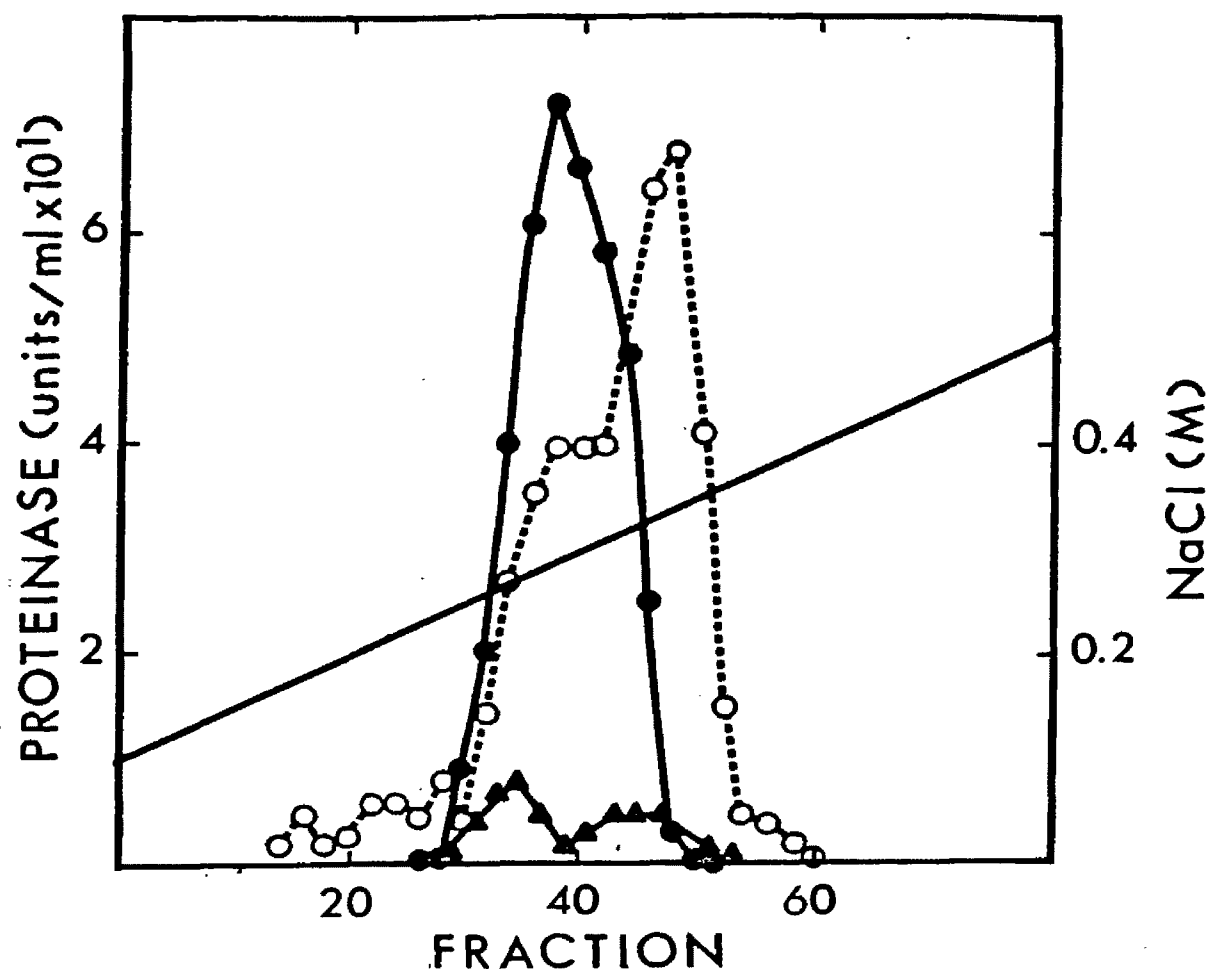
\*Acid Treated

Figure 10      The Isolation of Freeze-Thaw Activated Proteinase by DEAE Cellulose Chromatography.

Ten trays of vegetative amoebae of *D. discoideum* were harvested in 0.5 M sucrose. The washed cells were suspended to a cell density of  $5.0 \times 10^8$  cells per ml in 0.5 M sucrose. The cytosolic fraction was obtained by homogenization and centrifugation and concentrated to a volume of 10 ml. This preparation was equilibrated in 10 mM Tricine (pH 8.0), 0.1 M NaCl, 0.5 mM DTT and applied to a column (1.5 x 13 cm) of Whatman-DE-53 cellulose previously equilibrated in the above buffer. The column was eluted with buffer followed by elution with a 400 ml linear NaCl gradient from 0.1 M to 0.5 M. Five ml fractions were collected. The fractions were assayed in the following manner: 1) the fractions were assayed for esterase activity (▲▲). 2) Small aliquots (400 ul) of each fraction were frozen at  $-20^{\circ}\text{C}$  overnight, thawed and assayed for esterase activity (●●). 3) The fractions were then assayed in the presence of 1.3 M TCA-Na (pH 5.5) (○○).

Figure 11      The Isolation of Freeze-Thaw Activated Proteinase by Sephadex G-200 Chromatography.

Column fractions containing the freeze-thaw activated proteinase (from Figure 10) were pooled and concentrated to a volume of 4 ml. This preparation was applied to a column (2.6 x 53 cm) of Sephadex G-200 previously equilibrated in 10 mM Tricine (pH 8.0), 0.5 mM DTT and eluted with the same buffer. Five ml fractions were collected and assayed in a manner identical to that above: 1) The fractions were assayed for esterase activity (▲▲). 2) Small aliquots (400 ul) were frozen at  $-20^{\circ}\text{C}$  overnight, thawed and assayed for esterase activity (●●). 3) The fractions were then assayed in the presence of 1.3 M TCA-Na (pH 5.5) (○○).



Earlier studies (22) have demonstrated that the inhibition of Proteinase 1 can be reversed by incubation in the presence of the chaotropic agent Trichloroacetate (TCA·Na). In order to detect Proteinase 1, small volumes of the column fractions were assayed in the presence of TCA·Na buffered in Sodium Acetate at pH 5.5. The results showed that a peak of esterase activity separate from the freeze-thaw activated peak was unmasked. This peak corresponded to the elution characteristics of Proteinase 1. The results also revealed a partial unmasking of the freeze-thaw activated peak. During this study, esterase activity corresponding to Proteinase 1 and Proteinase 2 eluted from the column very close to each other. To obtain a preparation of the freeze-thaw activated proteinase free of Proteinase 1, the column fractions containing the freeze-thaw activated esterase were pooled and concentrated. This material was applied to a column of Sephadex G-200. The results of this chromatography (Figure 11) showed that the esterases corresponding to Proteinase 1 and Proteinase 2 were clearly separated. Again it was observed that TCA·Na treatment as well as freezing and thawing provided for the unmasking of the freeze-thaw activated proteinase. The fractions containing the freeze-thaw activated proteinase were pooled and concentrated to a volume of 8 ml and divided into two equal parts, A and B. Part A was stored frozen at  $-20^{\circ}\text{C}$ . Part B was mixed with an equal volume of glycerol and stored at  $-20^{\circ}\text{C}$ . Thawed aliquots of Part A represented the unmasked species of the freeze-thaw activated proteinase. It was predicted that since Part B was never frozen, the proteinase would remain in a masked state. This was indeed the case, suggesting that the physical event of freezing and not simply incubation at  $-20^{\circ}\text{C}$  was required for the unmasking

of the esterase.

#### VII. Analysis of the Freeze-Thaw Activated Proteinase.

Figure 12 compares the Sephadex G-200 profiles of masked and unmasked enzyme prepared as described in Section VI. The upper panel represented the Sephadex G-200 profile of the sample stored in glycerol and the lower panel represented the Sephadex G-200 profile of the unmasked species. The unmasked enzyme had a lower apparent molecular weight than the masked enzyme. This difference in molecular weight could be due to the release of a bound molecule upon freeze-thaw treatment.

The results of the Figures 10 and 11 supported the view that the cytosolic, freeze-thaw activated proteinase was unmasked in the presence of 1.5M TCA·Na. To determine the optimal concentration of TCA·Na required for unmasking, the masked proteinase was incubated at different concentrations of TCA·Na ranging from 0.5M to 1.5M. Figure 13 revealed that when the concentration of TCA·Na was less than 0.5M, there was no significant unmasking of the freeze-thaw activated proteinase. However, as the TCA·Na was increased, optimal unmasking of the esterase occurred at approximately 1.3M.

Figure 14 shows that the unmasking of cytosolic esterase by freeze-thaw treatments was influenced by pH. The masked enzyme was not significantly activated in acidic buffers. The optimal pH for the freeze-thaw activation of the enzyme was 7.0 - 8.0.

In addition to the unmasking of the cytosolic esterase by freeze-thaw and TCA·Na treatments, it has been found that the proteinase could also be activated in the presence of 0.1% SDS. Table 2 compares the

Figure 12      Sephadex G-200 Chromatography of Masked and Unmasked Freeze-Thaw Activated Proteinase.

The masked species of the isolated freeze-thaw activated proteinase (Figures 10 and 11) was applied to a column (1.7 x 40 cm) of Sephadex G-200 previously equilibrated in 10 mM Tricine (pH 8.0), 0.5 mM DTT and eluted with the same buffer. One ml fractions were collected. The collected fractions were frozen at -20°C overnight, thawed and assayed for esterase activity (Panel A).

The unmasked species of the proteinase was prepared prior to chromatography by freezing and thawing of the isolated masked species. This preparation was applied to the same column of Sephadex G-200 as described above and the fractions were assayed for esterase activity (Panel B).

The dashed arrow represented the location of Proteinase 1

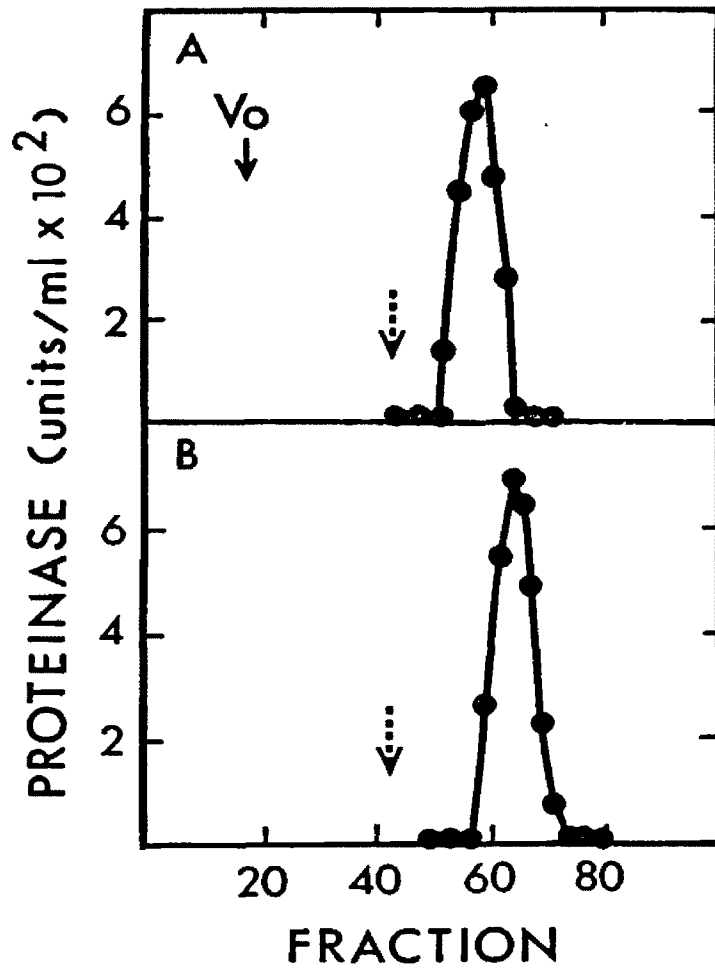
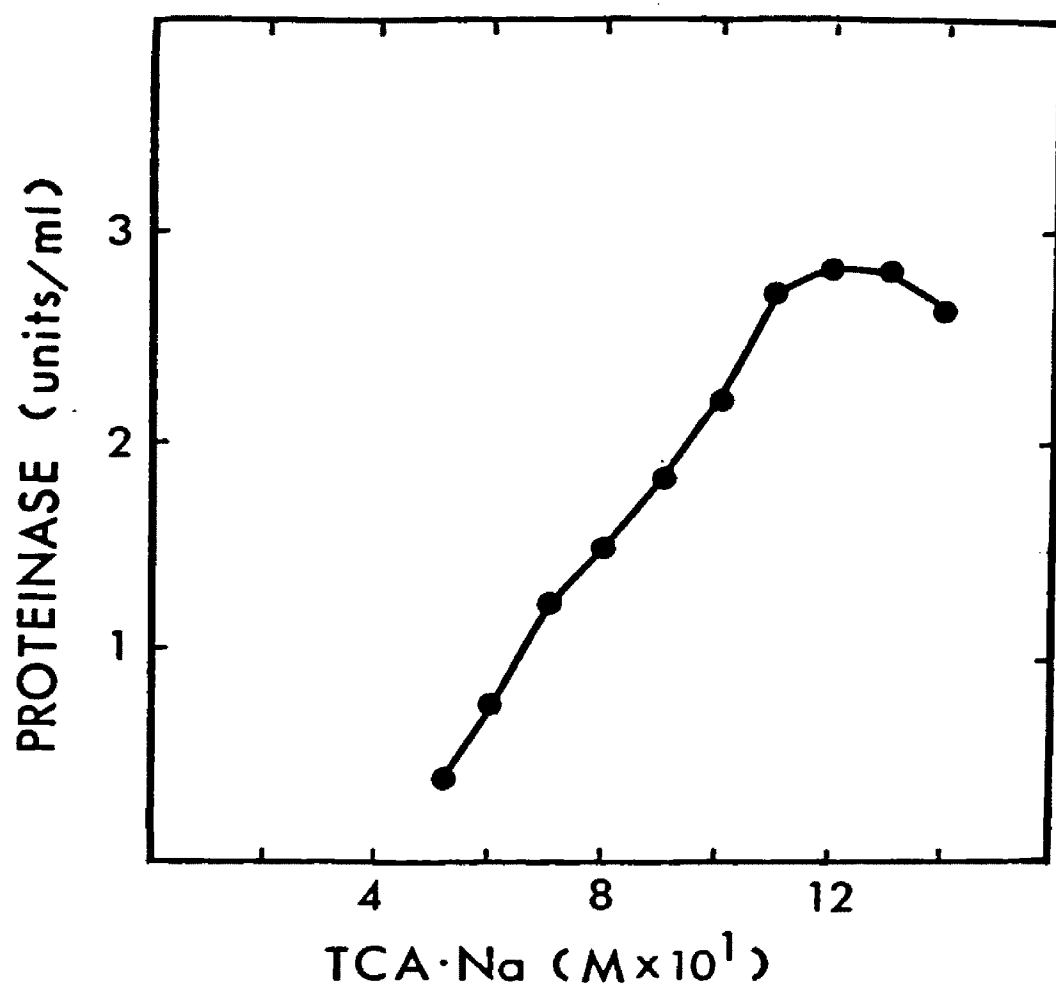


Figure 13      The Unmasking of the Freeze-Thaw Activated Proteinase  
by Trichloroacetate.

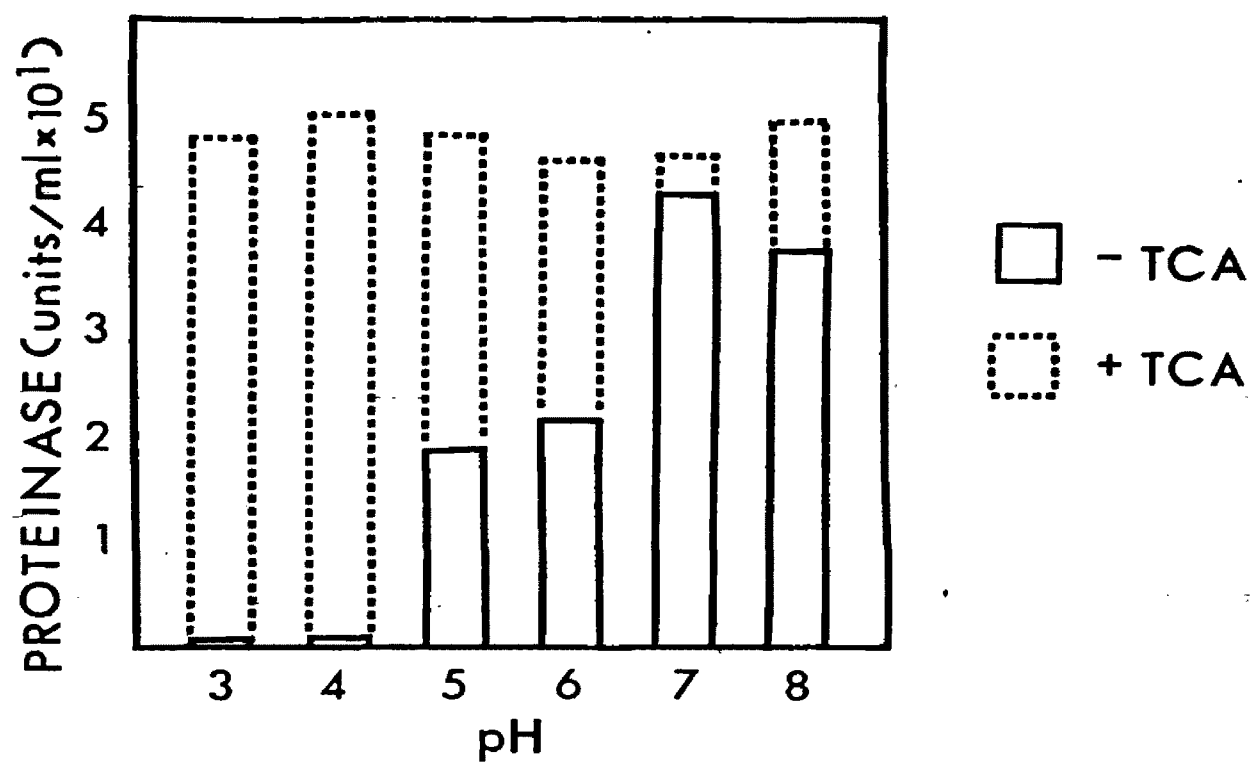
Small aliquots of the masked species of the isolated freeze-thaw activated proteinase were incubated in concentrations of TCA·Na (pH 5.5) ranging from 0.5 M to 1.4 M. These preparations were then assayed for esterase activity.





**Figure 14      The Effect of pH on the Unmasking of the Freeze-Thaw Activated Proteinase.**

The masked species of the freeze-thaw activated proteinase was incubated for 5 minutes in a series of Citrate-Phosphate buffers ranging in pH from 3.0 to 8.0. After incubation, the preparations were frozen at  $-20^{\circ}\text{C}$  overnight, thawed and assayed in the presence and absence of 1.3 M TCA·Na (pH 5.5).



**Table 2: The Unmasking of Freeze-Thaw Activated Proteinase by Various Treatments.**

<u>Treatment</u> <u>Treatment</u>	<u>Relative Activity</u> <u>(units/ml)</u>
1. No Treatment	0.18
2. Frozen-Thawed (F/T)	0.99
3. Acid Treatment (pH 3.0)	0.15
4. 1.3 M TCA	0.83
5. 0.1% SDS	1.02
6. F/T + Acid (pH 3.0) (2+3)	0.99
7. F/T + 1.3 M TCA (2+4)	1.04
8. F/T + 0.1% SDS (2+5)	0.90

extents of unmasking of this enzyme observed with these different treatments. Each treatment when applied individually appeared to activate the cytosolic esterase to the same extent. No cumulative effects were observed with the various combinations of treatments. That is, the esterase activity unmasked in combined treatments was no greater than that observed with individual treatments. As anticipated, acid incubation at pH 3.0 did not unmask the cytosolic proteinase.

#### VIII. Comparison of the Freeze-Thaw Activated Proteinase and Purified Proteinase 2.

The freeze-thaw activated esterase had chromatographic properties similar to those of Proteinase 2 from whole cell extracts. The purpose of this section was to compare these two enzymes based on their reactions with specific antisera. For these analyses, purified Proteinase 1, purified Proteinase 2 and the masked species of the freeze-thaw activated proteinase were first subjected to SDS-PAGE and then electroblotted to a nitrocellulose membrane. The nitrocellulose membrane was then probed with Anti-Proteinase 1-Affinity Purified antisera (see Materials). The results in Figure 15 showed that the freeze-thaw activated proteinase and purified Proteinase 2 had an identical molecular weight profile. The freeze-thaw activated proteinase had a major subunit of approximately 20 kilodaltons which matched that of the purified Proteinase 2 and also agreed with previous SDS-PAGE analyses (see Figure 2).

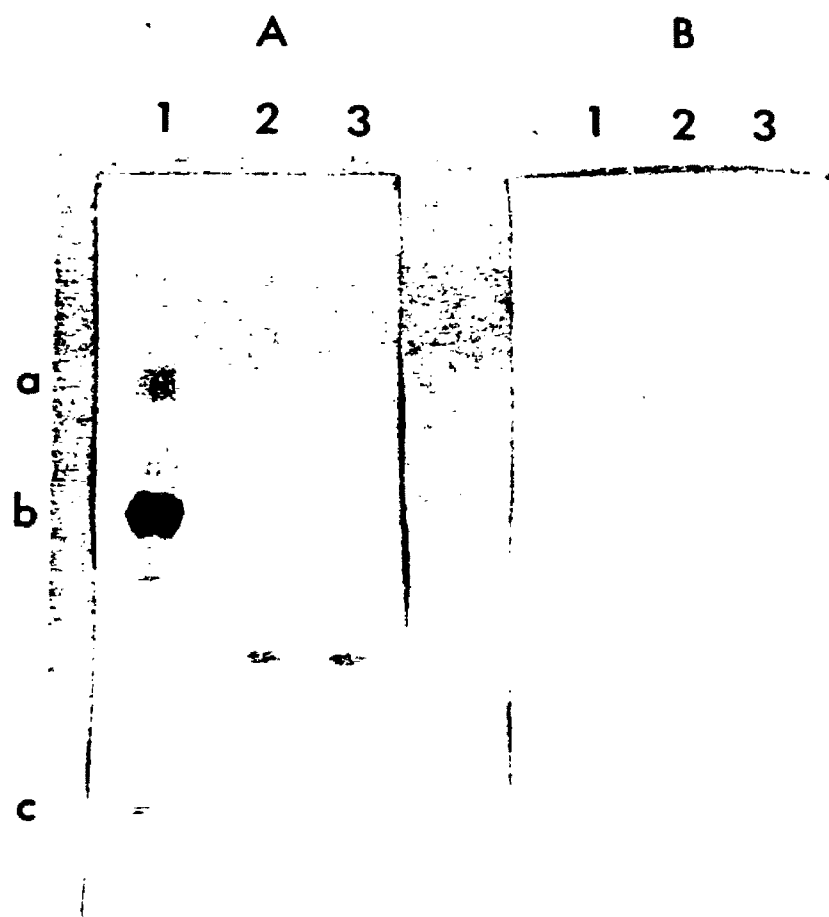
#### IX. Comparison of the Freeze-Thaw Activated Proteinase and Purified Proteinase 2 Using the Caseogram Method.

The freeze-thaw activated esterase was also compared to purified Proteinase 2 by exploiting the fact that the proteinases were caseolytic. Purified preparations of Proteinase 1, Proteinase 2 and the masked species

**Figure 15**      **Comparison of the Freeze-Thaw Activated Proteinase with Purified Proteinase 2 Using Specific Antisera.**

Purified samples of Proteinase 1, Proteinase 2 and the masked species of the freeze-thaw activated proteinase were subjected to 15% SDS-PAGE followed by electrophoretic transfer to a sheet of nitrocellulose membrane. The nitrocellulose was then reacted with a 1:600 of Anti-P1-N-Acetylglucosamine-1-Phosphate-Affinity Purified antisera (see Materials) (Panel A).

To examine the specificity of the antisera, a duplicate study was performed, however, the antisera was preabsorbed with 50 mM Uridine Diphosphate- N-Acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc was the ligand used to purify the antisera on an affinity column. As anticipated, no reaction occurred, confirming the fact that the antisera is specific for N-Acetylglucosamine-1-Phosphate residues esterified to Proteinase 1 and Proteinase 2 (Panel B).

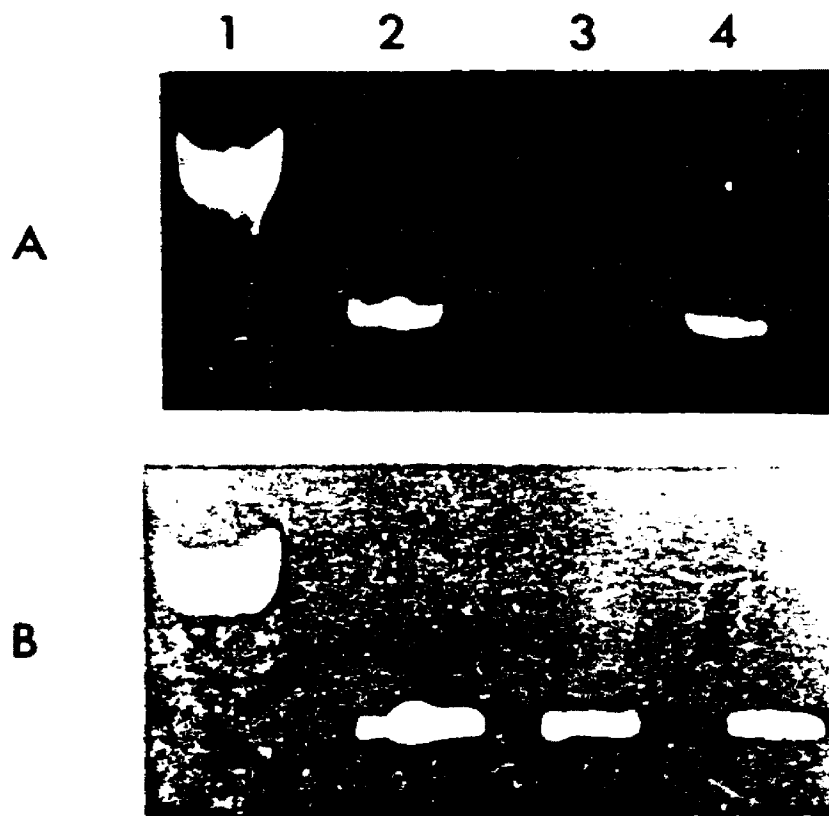


of the cytosolic proteinase were subjected to non-dissociating phosphate gel electrophoresis. By layering the gel on a casein-agarose plate, proteolytic activity was observed by the formation of precipitates in the casein. Panel A of Figure 16 shows that purified Proteinase 2 had greater mobility than Proteinase 1 in non-dissociating gels, similar to the results presented earlier in Figure 3. The results also show that the masked enzyme did not exhibit any caseolytic activity. In an effort to unmask the cytosolic esterase, the electrophoresis gel was carefully removed from the casein-agarose plate and incubated in 0.1% SDS buffered in 0.2M NaAc (pH 5.5) for 30 minutes. The gel was then layered onto a new casein-agarose plate. Panel B demonstrated that the masked esterase was activated and had an identical mobility of purified Proteinase 2



**Figure 16**      **Comparison of the Freeze-Thaw Activated Proteinase and Purified Proteinase 2 Using the Caseogram Method.**

Purified preparations of Proteinase 1 (Lane 1), Proteinase 2 (Lane 2) and the masked and unmasked species of the freeze-thaw activated proteinase (Lanes 3 and 4, respectively) were subjected to electrophoresis in a non-dissociating phosphate-buffered PAGE gel as described in the Methods section VII, A. The gel was removed from the electrophoresis apparatus and layered onto a casein-agarose plate. After incubation at room temperature for two hours, digestion of casein was observed by the formation of opaque precipitates in the casein. (Panel A). After development, the gel was removed from the casein-agarose plate and incubated in 0.2 M NaAc (pH 5.5) containing 0.1% SDS for 30 minutes. The gel was then removed from the SDS and layered onto a new casein-agarose plate and allowed to develop (Panel B).



## CHAPTER IV

### DISCUSSION

#### I. The Comparison of Purified Proteinase 2 and the Freeze-Thaw Activated Proteinase.

Previous studies have described the isolation of two major thiol endopeptidases referred to as Proteinase 1 and Proteinase 2 from whole cell extracts of D. discoideum. Proteinase 1 and Proteinase 2 shared many properties in common. They both digested several model protein substrates and had similar specificities for cleaving peptide bonds. Proteinase 2, however, was classified as a distinct enzyme from Proteinase 1 based on the following criteria: 1) during the purification of the proteinases, Proteinase 2 did not co-precipitate with protamine sulfate and nucleic acids whereas Proteinase 1 did precipitate. 2) Based on chemical analyses, Proteinase 2 contained ten fold less N-Acetylglucosamine-1-Phosphate than Proteinase 1. 3) Proteinase 2 had a lower apparent molecular weight than Proteinase 1 when the proteinases were compared by Sephadex G-200 chromatography and 4) Proteinase 2 required a lower concentration of salt for elution from an ion-exchange column.

The studies presented in this thesis originally set out to compare some of the electrophoretic properties of Proteinase 1 and Proteinase 2. SDS-PAGE revealed that Proteinase 2 had a single major polypeptide with a molecular weight of approximately 20 kilodaltons. In comparison, Proteinase 1 had three major subunits with molecular weights of 55, 39 and 15 kilodaltons. When the proteinases were subjected to non-dissociating PAGE, Proteinase 2 had a greater mobility than Proteinase 1. Immunological analyses, coupled with SDS-PAGE studies, demonstrated that Proteinase 2 contained sugar-phosphate determinants similar to those found in

Proteinase 1. This suggested a common pattern of glycosylation for Proteinase 1 and Proteinase 2.

The compartmentations of Proteinase 1 and Proteinase 2 were examined by analyzing the esterase activity present in cytosolic and vacuolar fractions. Sephadex G-200 chromatography of these fractions demonstrated that the vacuolar fraction contained only active Proteinase 1 and no detectable Proteinase 2. The majority of Proteinase 1 was located in the vacuolar fraction in an active form. The remaining Proteinase 1 was present in the cytosolic fraction in a masked state. Data were presented (Table 1) which suggested that the presence of Proteinase 1 in the cytosolic fraction was a result of the leakage of Proteinase 1 from the cell vacuoles into the cytosol during homogenization. The escaped enzyme was then rendered inactive by an inhibitor specific for Proteinase 1 located only in the cytosol. The cytosolic fraction contained no significant esterase. This result prompted us to evaluate if Proteinase 2 occurred in a masked state in cell homogenates. Initial observations revealed that the esterase activity present in the cytosolic fraction increased 4-5 fold when subjected to freeze-thaw treatment at  $-20^{\circ}\text{C}$ . Eventually it was determined that a freeze-thaw activated proteinase, present only in the cytosolic fraction, had properties very similar to those of purified Proteinase 2. One of the major objectives of the subsequent studies was to determine if the freeze-thaw activated proteinase was the enzyme referred to earlier as Proteinase 2. It was demonstrated that the freeze-thaw activated proteinase had identical properties to purified Proteinase 2 based on the following: 1) The relative elution volumes during Sephadex G-200 chromatography of the freeze-thaw activated

proteinase and purified Proteinase 2 were identical. 2) Both proteinases eluted from an ion-exchange column at the same salt concentration.

3) The freeze-thaw activated proteinase had an identical mobility to purified Proteinase 2 in non-dissociating PAGE gels and 4) The immunological reactions of both proteinases was identical when reacted with antisera specific for the N-Acetylglucosamine residues present on both purified Proteinase 1 and Proteinase 2.

## II. The Mechanism of Unmasking of Proteinase 2.

Studies of the Proteinase 1-Inhibitor 1 complex (22) have demonstrated that the inhibition of Proteinase 1 was due to a heat-stable, acid labile protein inhibitor. The mechanism of masking of Proteinase 2 has not yet been elucidated. However, two observations support the idea that masking may involve the association of an inhibitor with Proteinase 2. First, the masked form of Proteinase 2 had a higher molecular weight than unmasked Proteinase 2 when comparing the relative elution volumes during Sephadex G-200 chromatography. The differences in molecular weight between masked and unmasked enzyme could be due to the presence of an inhibitor. Secondly, agents known to dissociate the Proteinase 1-Inhibitor 1 complex (i.e. TCA, SDS) unmask Proteinase 2 to the same extent as freezing and thawing. Therefore, the unmasking of Proteinase 2 by freezing and thawing could involve the disruption of a Proteinase 2-Inhibitor 2 complex. However, if Proteinase 2 existed in the cytosolic fraction as an enzyme-inhibitor complex, it is doubtful that this inhibitor was identical to the inhibitor which was involved in the masking of proteinase 1. This view follows from the fact that there were essential

differences between the masking of Proteinase 1 and the masking of Proteinase 2. First, partially purified masked Proteinase 2 was not activated by the acid treatment which was shown to inactivate Proteinase 1 inhibitor. Secondly, masked Proteinase 1 was not activated by the freeze-thaw treatment which was demonstrated to unmask Proteinase 2

Another possibility for the mechanism of masking of Proteinase 2 would be that Proteinase 2 existed as a zymogen in the cytosolic fraction and that catalytic cleavage was required for activation. Several examples of this type of enzyme activation are known. The serine proteinase Trypsin, for example, is secreted from the pancreas into the small intestine as the inactive zymogen Trypsinogen (28). Trypsinogen then undergoes a catalytic cleavage by the enzyme Enteropeptidase which removes a hexapeptide thus converting Trypsinogen to Trypsin. This type of mechanism of action has not been totally ruled out for Proteinase 2. The freeze-thaw treatment of masked Proteinase 2 generated an active enzyme with a lower molecular weight. Freezing and thawing could alter the conformation of Proteinase 2 in such a way that the proteinase can act upon itself and cleave a peptide bond. It is doubtful that a second enzyme was involved in the activation of Proteinase 2, since the masked species of Proteinase 2 can be isolated in a pure state and freeze-thaw activation still occurred.

### III. The Significance of Masking of Proteinase 1 and Proteinase 2.

As discussed previously (Introduction, Section III) the presence of cytosolic inhibitors has been demonstrated in yeast and rat liver systems. These inhibitors generally served a function related to a stress

response in the cell. One proposed function of the inhibitors was to protect cytosolic proteins from unwanted proteolysis due to the leakage of proteinases from the vacuolar compartment. This was also a reasonable assumption to make in D. discoideum. It has been shown that during the homogenization procedure used to separate cytosolic and vacuolar compartments, Proteinase 1 containing vacuoles were partially disrupted and Proteinase 1 escaped into the cytosolic compartment. The proteolytic activity of Proteinase 1 was suppressed, however, because the enzyme became bound to its inhibitor.

An entirely different area in which proteinase inhibitors may function was in transport. Lenney (17) has suggested the possible role of cytosolic inhibitors as transport molecules of newly synthesized proteinases from cytosolic polysomes to their vacuolar compartments. This idea was also a possibility in D. discoideum. This pathway has not yet been elucidated, but perhaps the proteinase inhibitors serve a role. The inhibitors could bind to proteinases recently synthesized on cytosolic polysomes and direct the compartmentation of proteinases to either remain in the cytosol (Proteinase 2) or be transported to vacuoles (Proteinase 1). If this is the case, then the inhibitors would be serving a two fold function: 1) To prevent unwanted proteolysis of cytosolic proteins and 2) To transport proteinases to various cell compartments.

Two paradoxes were evident from the results. First, Proteinase 2 was present only in the cytosolic fraction of vegetative amoebae and in a masked state. In addition to these observations, other studies have shown\* that Proteinase 2 is secreted during differentiation. Therefore,

\*Unpublished results

it appeared that Proteinase 2 did not have a function during normal growth and differentiation. Cytosolic Proteinase 2 could function in the degradation in cytosolic proteins generated by a stress condition. Freezing and thawing may induce abnormal proteins in the cytosol. The activation of Proteinase 2 in response to this condition may serve to degrade these proteins.

Secondly, these studies have shown that Proteinase 1 and Proteinase 2 shared a common pattern of glycosylation but different compartmentations. This suggested an unusual pathway for the trafficking of these enzymes. It is generally accepted that all lysosomal enzymes which contain N-linked oligosaccharides were first synthesized on ribosomes of the endoplasmic reticulum (ER). As the protein is translated, it becomes inserted into the lumen of the ER where it is immediately glycosylated (29). These glycoproteins are modified to a variety of products in the ER and processing continues in the Golgi. The enzymes are then transported to the lysosomes by an unknown mechanism. The O-linked sugar in Proteinase 1 and Proteinase 2 suggested that glycosylation occurred in compartments separate from those of N-linked glycosidases.

The enzyme involved in the glycosylation of Proteinase 1 and Proteinase 2 may be affected by factors which influence the synthesis of greater amounts of Proteinase 1 or Proteinase 2. For example, it has been shown (14) that with cells grown axenically in liquid culture, that the synthesis of Proteinase 2 predominates over Proteinase 1. It was possible that nutritional requirements dictated which proteinase would be produced. Earlier studies (7) demonstrated that Proteinase 1 and Proteinase 2 differ in their extents of phosphoglycosylation. Proteinase 2 contained



10 fold less N-Acetylglucosamine-1-Phosphate than Proteinase 1. In addition to these differences in glycoposphorylation, further modification of the proteinases must be involved. SDS-PAGE data have shown that Proteinase 1 was a polymeric enzyme with three major subunits which totalled in molecular weight to approximately 110 kilodaltons. Proteinase 2 had a single major polypeptide with a molecular weight of approximately 20 kilodaltons. It was possible that Proteinase 1 and Proteinase 2 were the result of a common gene product. The extent of conjugation with N-Acetylglucosamine-1-Phosphate could be a signalling factor determining whether or not the protein is cleaved. That is, Proteinase 1 has a ten fold greater content of N-Acetylglucosamine-1-Phosphate and therefore has a higher net negative charge. Proteinase 2 would be less negative which may make it more susceptible to some enzyme and therefore was modified to a lower molecular weight enzyme.

## CHAPTER V

## SUMMARY

It has been concluded from these results that:

1. Proteinase 1 and Proteinase 2 had different electrophoretic properties in both dissociating and non-dissociating PAGE gels. During dissociating PAGE, Proteinase 1 resolved as three major subunits of 55, 39 and 15 kilodaltons. Proteinase 2 resolved as a single polypeptide with a molecular weight of approximately 20 kilodaltons. During non-dissociating PAGE, both Proteinase 1 and Proteinase 2 resolved as single bands. Proteinase 2 had a greater mobility than Proteinase 1.
2. Proteinase 1 and Proteinase 2 had different compartmentations in vegetative cells. Proteinase 1 was found to be localized in an active form in the vacuolar fraction. Proteinase 2 was found to be exclusive to the cytosolic fraction and was present only in a masked state.

From these results it has been suggested that:

1. Proteinase 2 may function in the cell in a stress response to degrade abnormal cytosolic proteins generated during a stress condition.
2. Inhibitors of Proteinase 1 and Proteinase 2 may function in the transport of the newly synthesized proteinases from cytosolic polysomes to their cellular compartments.

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